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14. ABSTRACT Vitamin D is an important hormone that affects the incidence and progression of many malignancies including prostate cancer (PCa). 1,25-dihydroxyvitamin D_3 (calcitriol), the active form of vitamin D_3, inhibits growth and stimulates differentiation of PCa cells. We study established human PCa cell lines to elucidate the molecular pathways of calcitriol actions. These pathways are varied and appear to be cell-specific. To identify novel therapeutic targets for the treatment of PCa, we have used cDNA microarray analysis to ascertain additional genes regulated by calcitriol. Several potentially useful target genes have emerged from these studies. In this project we analyze two new target genes, both involved in prostaglandin (PG) metabolism. PGs are implicated in stimulating the development of PCa, associated with the progression of PCa, tumor invasiveness and tumor grade. Prostatic PGs are formed by the action of the cyclooxygenase enzyme COX-2. The first step in PG inactivation is mediated by 15-hydroxyprostaglandin dehydrogenase (15-PGDH). We found that calcitriol down-regulates the expression of COX-2 and up-regulates 15-PGDH. There is much current interest in the use of second-generation COX-2 inhibitors, to prevent and/or treat PCa, due to their ability to inhibit growth and induce apoptosis. Moreover, 15-PGDH has recently been proposed as a tumor suppressor. The actions of calcitriol to induce 15-PGDH and inhibit COX-2, constitute a pathway to reduce and/or remove active PGs thereby diminishing PCa proliferation. Combination therapy of LNCaP cells with calcitriol and COX-2 inhibitors revealed synergistic growth inhibition. In combination, calcitriol and COX-2 inhibitors allowed the use of reduced doses of both drugs that still resulted in enhanced anti-proliferative activity. These findings suggest that therapy combining calcitriol and COX-2 inhibitors will increase efficacy while decreasing side-effects. We propose that this combination of already approved drugs can be brought to clinical trial swiftly. In conclusion, our research is directed at understanding the mechanisms of calcitriol action in prostate cells with the goal of developing treatment strategies to improve PCa therapy. The ability of calcitriol to inhibit PG synthesis and stimulate PG destruction appears to be an additional pathway by which calcitriol can enhance PCa therapy.

15. SUBJECT TERMS
Prostate cancer (PCa), 1,25-dihydroxyyvitamin D_3 (calcitriol), prostaglandins (PGs), 15-hydroxyprostaglandin dehydrogenase (15-PGDH), cyclooxygenase enzyme COX-2

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INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed malignancy and the second leading cause of cancer death in North American men [**1**]. According to the American Cancer Society more than 232,000 men will be diagnosed with PCa in 2005 and approximately 10% of these men will die of the disease [**1**]. Primary therapy for PCa involves the removal of the prostate by surgery or radiation therapy. Unfortunately, after initial treatment PCa often recurs. Androgens regulate normal prostate development and growth. Surgical or medical androgen deprivation has been used as the standard treatment for PCa that fails primary therapy [**2, 3**]. Although there is a good initial response to androgen ablation in most men, tumors will progress to androgen independence resulting in death [**4**] since there is currently no adequate treatment for this advanced disease.

Our current investigation is aimed at the development of new therapies to treat PCa. 1α,25-Dihydroxyvitamin D_3 (calcitriol), the hormonally active form of vitamin D, is a promising new therapeutic agent for PCa therapy [**5-15**].

Calcitriol effects on PCa

Calcitriol is a steroid hormone known as the major regulator of calcium homeostasis and bone mineralization [**16, 17**]. However, data accumulated over the past 25 years indicate that calcitriol and its analogs have potent anti-proliferative and pro-differentiation actions in a number of malignancies including PCa [**15, 18-20**]. The anti-proliferative action of calcitriol has been documented in several PCa cell lines [**6, 8, 21, 22**] as well as in primary cultures of normal and cancer cells derived from surgical specimens taken from men with PCa [**23, 24**]. The inhibition of PCa cell growth is seen in both androgen-dependent and -independent PCa cells [**25, 26**]. A pilot study from our group provided preliminary evidence that calcitriol effectively slows the rate of PSA rise in PCa patients with early recurrent PCa [**27**]. Recent trials using intermittent high doses of calcitriol in combination with chemotherapy have shown great promise in prolonging survival and delaying time to progression in men with androgen-independent PCa [**13, 15**]. Many pharmaceutical companies are attempting to design calcitriol analogs with increased potency and less tendency to cause hypercalcemia, the only side-effect of calcitriol therapy [**28**]. We believe that calcitriol or a new analog will prove to be a very useful adjunct for the therapy of both androgen-dependent and -independent PCa.

Given the potential utility of calcitriol in treating and/or preventing PCa, understanding the molecular basis of calcitriol-mediated growth inhibition and the signaling pathways involved in it will fully define its therapeutic potential as well as allow the development of better therapeutic approaches to treat PCa progression. Moreover, uncovering the targets of calcitriol action will help develop a combination therapy that have both the same targets to enhance separate actions and/or aiming different molecules to amplify the spectrum of action mechanisms that each drug has alone.

Mechanisms of action of Calcitriol on PCa

Calcitriol exerts its action through the activation of its nuclear receptor, the vitamin D receptor (VDR). After hormone binding, VDR heterodimer attaches to DNA sequences known as vitamin D response elements (VDRE) in the promoter regions of target genes. This calcitriol-VDR complex then recruits co-activator proteins that stimulate the transcriptional apparatus to induce the expression of the target gene. A number of important pathways have been shown to have a role in calcitriol-mediated growth inhibition. One primary

mechanism of calcitriol action is to induce cell cycle arrest in the G1/G0 phase by increasing the expression of genes like insulin-like growth factor binding protein-3 (IGFBP-3) and p21, and to induce apoptosis by down-regulating the activity of anti-apoptotic genes, like bcl-2, [**5-10, 14**].

New targets of calcitriol in PCa

Using cDNA microarray analysis to study the alteration in gene expression elicited by the treatment of PCa cell lines with calcitriol, we have recently found 28 genes regulated by the hormone [**29**]. Among the up-regulated genes is NAD+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH). This enzyme is also induced by calcitriol in primary cultures of normal prostatic epithelial cells [**30**]. Interestingly, calcitriol also induces the expression of 15-PGDH in human neonatal monocytes [**31**]. We also found the down-regulation of various genes, including the prostaglandin-endoperoxide synthase-2, or cyclooxygenase-2 (COX-2) [**29**]. COX-2 is an enzyme involved in prostaglandin (PG) synthesis, while 15-PGDH is the primary enzyme responsible for PG catabolism.

Role of COX-2 activity in PCa

PGs are long-chain oxygenated polyunsaturated fatty acids derived from arachidonic acid (AA). COX or cyclooxygenases, are responsible for the synthesis of PG precursor PGH_2, which will latter be converted to each kind of PG by specific synthases [**32**]. PGs are implicated in stimulating proliferation of many cancers including PCa [**33**]. Many, yet not all, studies have concluded that COX-2 is over-expressed in PCa when compared with normal prostate [**34, 35**]. *In vitro* studies using the androgen-dependent -independent PCa cell lines showed that both express detectable amounts of COX-2 and secrete PGE_2 [**36**]. COX-2 is proposed to induce tumorigenesis in PCa by various mechanisms: (1) induction of cell proliferation [**37**], (2) decreased apoptosis [**38**], (3) increased angiogenesis [**39**]; (4) increased tumor invasiveness [**40**]; and (4) decreased immune surveillance [**41**]. Non-steroidal anti-inflammatory drugs (NSAIDs), known inhibitors of COX activity and therefore of PG synthesis, have been shown to decrease growth in PCa cells *in vitro* and *in vivo* [**33, 38, 42**]. Data suggest that PGE_2 has a specific role in the maintenance of human cancer cell growth and that the activation of COX-2 expression depends primarily upon newly synthesized PGE_2 through positive feedback [**33, 43**]. Knockout of the COX-2 gene generates a marked reduction in the number and size of intestinal polyps in a murine model of human familial adenomatous polyposis [**44**]. Taken together, these data indicate that COX-2 and/or their prostaglandin products play a role in the malignant transformation of the prostate.

The role of 15-PGDH in cancer.

15-PGDH is a key metabolic enzyme initiating the catabolic pathway of biologically active PGs producing inactive keto-derivatives [**45**]. Given that COX-2 initiates PG synthesis and 15-PGDH catalyses PG degradation, COX-2 and 15-PGDH are functional antagonists. Three pieces of evidence indicate that the concomitant over-expression of COX-2 and under expression of 15-PGDH have a role in tumor progression. First, microarray data analysis indicated a down regulation of 15-PGDH in colon [**46**] and lung [**47**] cancers when compared to normal tissues. Second, when colon epithelial cells are chronically treated with the well established tumor suppressor TGF-β [**48**] 15-PGDH gene expression is induced [**46**]. Thirdly, 15-PGDH seems to have tumor suppressor effects. When athymic nude mice were injected with cancer cells transiently over-expressing 15-PGDH, there was a substantial decrease in the induction of tumor growth when compared to wild type cells [**47**].

We have already demonstrated that calcitriol stimulates the expression of 15-PGDH (see Cancer Research paper in Appendix), we hypothesize that this action is related to the anti-proliferative activity of calcitriol. A rise in the activity of 15-PGDH would reduce the levels of biologically active PGs. We would expect calcitriol growth-inhibition to be mediated, at least partially, by the induction of 15-PGDH and the inhibition of COX-2 [**49**].

The role of NSAIDs in PCa.

Initially, the interest in the role of COX enzymes in the development of cancer came from the observation that patients taking NSAIDs have a lower risk of developing cancer PCa [**41, 50**]. NSAIDs prevent PG synthesis and prevent PGs from exerting their biological actions [**51**]. Inhibition of COX-2 by NSAIDs appears to provide a beneficial therapeutic action. Interestingly, NSAIDs also regulate 15-PGDH levels in some cells [**52, 53**].

Our hypothesis is that calcitriol regulation of PG metabolism (induction of 15-PGDH and inhibition of COX-2) is another pathway to remove active PGs that may help to diminish PCa proliferation. In this way, calcitriol and NSAIDs would have the same ultimate effect. Unfortunately, NSAID use has shown some secondary side-effects including increased risk of heart attacks, stroke, sudden death, blood clots, stomach and intestinal bleeding, kidney problems including acute kidney failure and worsening of chronic kidney failure [**54**]. On the other hand, calcitriol also has secondary effects, namely hypercalcemia. However, this effect can be diminished with intermittent administration of calcitriol [**15**] or with the use of the new analogs of calcitriol [**28**]. To avoid such unwanted actions we would resort to the combination of NSAIDs and calcitriol. We predict that the combination therapy could allow the use of lower doses of both drugs thus reducing their individual side-effects.

Given the induction of 15-PGDH expression and the inhibition of COX-2 expression by calcitriol, both NSAIDs and calcitriol would have the same effect, to reduce the pool of active PGs. To us, this strongly suggests that induction the regulation of PG-related metabolic genes by calcitriol contributes to its anti-proliferative activity. It also suggests a possible synergistic action of calcitriol and NSAID treatment to prevent cancer cell proliferation. We hypothesize that the role of calcitriol in the general metabolism of these eicosanoids, by induction of 15-PGDH and the inhibition of COX-2 expression, contributes to the anti-PCa action of calcitriol. By inhibiting COX-2 and stimulating 15-PGDH expression, calcitriol would decrease the levels of biologically active PGs in PCa cells and thereby reduce the proliferative stimulation of PGs, much like the NSAIDs. Our finding that calcitriol stimulates the expression of 15-PGDH and inhibits COX-2 assume greater significance considering the putative actions of PGs on PCa cells.

BODY

The main findings of this ongoing project are fully described in the Cancer Research paper: Regulation of prostaglandin metabolism by calcitriol attenuates growth stimulation in prostate cancer cells [**55**] (Appendix II, Publications).

Calcitriol actions on the prostaglandin pathway in PCa cells

PGs synthesis is initiated by cyclooxygenases (COX) -1 and -2 [**32**] and their degradation is mediated by 15-prostaglandin dehydrogenase (15-PGDH) [**45**]. Elevated levels of prostaglandins are associated with increased cancer risk and more aggressive cancer. Calcitriol, the active form of vitamin D, is potentially useful in cancer treatment by interfering with PG metabolism and actions.

Our microarray data indicated that calcitriol increased the expression of 15-PGDH and significantly decreased the expression of COX-2 in LNCaP human PCa cells [**29**]. We hypothesize that this dual action of calcitriol would reduce the levels of biologically active PGs in PCa cells, thereby decreasing the proliferative stimulus for PCa growth.

Our experiments showed that calcitriol inhibits prostaglandin actions in PCa cells by three mechanisms: stimulating the expression of prostaglandin catabolizing enzyme 15-PGDH, decreasing the expression of prostaglandin synthesizing enzyme COX-2 and inhibiting EP2 and FP prostaglandin receptor expression [**55**]. We also observed a decrease in levels of PGs in the conditioned media of LNCaP cells treated calcitriol. We suspect that this was the result of the dual effect of calcitriol on the expression of PG metabolic enzymes [**55**]. Furthermore, the combination of calcitriol and various non-steroidal anti-inflammatory drugs (NSAIDs) produced synergistic inhibition of PCa cell growth at 2 to 10 lower concentrations of the drugs needed to achieve the same effect alone. The findings suggest that calcitriol and NSAIDs may be a useful combination for chemotherapy PCa [**55, 56**].

KEY RESEARCH ACCOMPLISHMENTS

Training accomplishments

Dr. Moreno has spent her time performing experiments in the laboratory of Dr. David Feldman, under the guidance of Dr. Feldman and Dr. Aruna Krishnan, a very experienced Research Scientist.

Dr. Moreno has periodically presented her data to the group of collaborators from the laboratories of Dr. Feldman and Dr. Donna Peehl, a long time collaborator, at the weekly lab meetings. Dr. Moreno has made formal presentations to the Stanford Prostate Cancer Group and to the Department of Urology Research Conference.

Dr. Moreno attended classes in "Responsible Conduct of Research", once a week for eight consecutive weeks in the period of September-November, 2004. The course was comprised of lectures on environmental health and safety, use and protection of human subjects and lab animals, conflicts of interest, publication, intellectual property and data, error, negligence or misconduct and response to violations of ethical standards. She has also gained experience in prostate cancer biology, normal and abnormal prostate cell function, prostate cancer therapy, chemoprevention strategies and the design of new treatment therapies to delay or prevent prostate cancer progression. The methods employed focus on hormone action, nuclear receptors, and regulation of target gene transcription and protein expression as well as metabolic studies of enzymatic activity and regulation of gene product concentration. The methods applied included cell culture, gene expression analysis by real time RT-PCR, which represents a completely new technique for her, Western blot, gene regulation studies, transfection and reporter gene assays.

During the period of September 2004 to September 2005, Dr. Moreno has completed the publication of three research papers and one review (see Appendix I-Biosketch). Two papers emanate from her previous work in Mexico, where she is originally from and had her doctoral training. The third paper in *Cancer Research* is the culmination of the first part of the current project. Dr. Moreno has also written 2 reviews, on published in the *Journal of Steroid Biochemistry and Molecular Biology* and the second in press in *Anticancer Research.* The results of the current project have also been presented at several meetings: (1) by Dr. Feldman at the 10th Prouts Neck Meeting on Prostate Cancer, in Maine in October 2004, (2) at the Vitamin D Symposium on Cancer, organized by the NIH: Chemoprevention & Cancer Treatment: Is there a role for vitamin D, 1α,25(OH)2-vitamin D3 or new analogs (deltanoids)", in November, 2004 in Bethesda, Maryland, (3) at the 2nd Int. Symposium on Vitamin D Analogs in Cancer Prevention and Therapy. May 2005, Lübeck, Germany and (4) by Dr. Moreno at the Endocrine Society's 87th Annual Meeting, in San Diego California in June 2005.

Research accomplishments

The major part of the scientific accomplishments is described in detail in the paper published in Cancer Research (Vol. 65: 7917, Sept. 2005), found in the Appendix.

Calcitriol exhibits anti-proliferative and pro-differentiation effects in prostate cancer. Our goal is to further define the mechanisms underlying these actions. We studied established human prostate cancer cell lines and showed that calcitriol interferes with the metabolism of prostaglandins (PGs), known stimulators of prostate cell growth in three ways:

- Calcitriol significantly repressed the mRNA and protein expression of prostaglandin endoperoxide synthase/cyclooxygenase-2 (COX-2), the key PG synthesis enzyme.
- Calcitriol up-regulated the expression of 15-hydroxyprostaglandin dehydrogenase, the enzyme initiating PG catabolism.
- We found that this dual action was associated with decreased prostaglandin E2 secretion into the conditioned media of prostate cancer cells exposed to calcitriol.
- Calcitriol also repressed the mRNA expression of the PG receptors EP2 and FP, providing a potential additional mechanism of suppression of the biological activity of PGs.
- Calcitriol treatment attenuated PG-mediated functional responses, including the stimulation of prostate cancer cell growth and the up-regulation of PG target genes as *c-fos*.
- The combination of calcitriol with NSAIDs synergistically acted to achieve significant prostate cancer cell growth inhibition at approximately 2 to 10 times lower concentrations of the drugs than when used alone.
- The regulation of PG metabolism and biological actions constitutes a novel pathway of calcitriol action that may contribute to its anti-proliferative effects in prostate cells.
- We propose that a combination of calcitriol and nonselective NSAIDs might be a useful chemo-preventive and/or therapeutic strategy in men with prostate cancer, as it would allow the use of lower concentrations of both drugs, thereby reducing their toxic side effects.

REPORTABLE OUTCOMES

Publications:

- Jaramillo, B.E., Ponce, A., **Moreno, J.**, Betanzos, A., Huerta, M., Lopez-Bayghen, E., Gonzalez-Mariscal, L. 2004. Characterization of the tight junction protein ZO-2 localized at the nucleus of epithelial cells. *Exp. Cell Res.* **297**: 247-258.
- Shoshani, L., Contreras, R.G., Roldan, M.L., **Moreno, J.**, Lazaro, A., Balda, M.S., Matter, K., Cereijido, M. 2005. The polarized expression of Na+,K+-ATPase in epithelia depends on the association between beta-subunits located in neighboring cells. *Mol. Biol. Cell.* **16**: 1071-1081.
- Moreno, J., Krishnan, A.V., Feldman, D. 2005. Molecular mechanisms mediating the anti-proliferative effects of Vitamin D in prostate cancer. *J. Steroid. Biochem. Mol. Biol.* Jul 14; [Epub ahead of print].
- **Moreno J.**, Krishnan A.V., Swami S., Nonn L., Peehl D.M., Feldman, D. 2005. Regulation of prostaglandin metabolism by calcitriol attenuates growth stimulation in prostate cancer cells. *Cancer Res.* **65**: 7917-7925.
- **Moreno, J.**, Krishnan, A.V., Feldman, D. 2005. Pathways mediating anti-proliferative actions of calcitriol in prostate cancer. *Anticancer Res.* In press.

Scientific Meetings:

- Feldman, D., **Moreno, J**., Krishnan A.V. Pathways mediating the growth inhibitory actions of Vitamin D in prostate cancer. 10th Prouts Neck Meeting on Prostate Cancer, Prouts Neck, Maine October 2004,
- Feldman, D., **Moreno, J**., Krishnan A.V. Pathways mediating the growth inhibitory actions of Vitamin D in prostate cancer. Vitamin D symposium: Cancer Chemoprevention & Cancer Treatment: Is there a role for vitamin D, 1α,25(OH)[**26**]2-vitamin D3 or new analogs (deltanoids)" November, 2004, Bethesda, Maryland.
- **Moreno, J**., Krishnan, A.V., Feldman, D. Pathways mediating the growth inhibitory actions of Vitamin D in prostate cancer. Abstracts of the 2nd Int. Symposium on Vitamin D Analogs in Cancer Prevention and Therapy. May 2005, Lübeck, Germany. *Anticancer Res.* 25. 2290.
- **Moreno J.**, Krishnan AV, Feldman D. Regulation of prostaglandin metabolism by calcitriol: Potential role in the treatment of prostate cancer. Endocrine Society's 87th Annual Meeting, in San Diego California, June 2005.

Pending accomplishments

Task III: **To test the potential additive effects of calcitriol and NSAID treatment.** (Months 13-16).

a) We will analyze the effects of NSAIDs and calcitriol individually and in combination on key genes known to respond to calcitriol including androgen receptor, prostate-specific antigen, and those genes involved in the signaling pathway mediating the calcitriol anti-proliferative actions including cyclin-dependent kinase inhibitor p21, insulin-like factor receptor binding protein (IGFBP-3) and the apoptosis inhibitor bcl-2.

b) We will measure the levels of PGs in conditioned medium from cells treated separately or simultaneously with calcitriol and NSAIDs.

Task IV: **To ascertain the role of 15-PGDH induction and COX-2 inhibition in the anti-proliferative action of calcitriol.** (Months 17-24).

a) We will develop an appropriate protocol to abrogate the activity of 15-PGDH in PCa cells by antisense and/or siRNA, to determine the extent of the effect of 15-PGDH inhibition on the total calcitriol anti-proliferative activity.

b) We will determine the silencing of 15-PGDH in enzymatic activity assays.

c) We will study the actions of calcitriol on cell growth in the absence of 15-PGDH.

d) We will stably transfect a human COX-2 gene expression vector into PCa cells and then, we will investigate the effect of calcitriol on the growth of PCa cells overexpressing COX-2 to determine the role of COX-2 repression on calcitriol-mediated growth inhibition.

We anticipate a period of 12 months dedicated to the completion of this second part of the project. All the Specific Aims will be covered in sequence.

CONCLUSIONS

Our research is directed at understanding the molecular mechanisms of the anti-proliferative activity of calcitriol in prostate cells with the goal of developing strategies to improve PCa treatment. Using cDNA microarrays we have recently found that calcitriol modulates the expression genes involved in PG metabolism. Calcitriol reduces the expression of COX-2 gene, the enzyme that catalyzes PG synthesis and up-regulates the expression of 15-PGDH gene, the enzyme involved in PG inactivation. In the current project we found that calcitriol acts by three separate mechanisms: decreasing COX-2 expression, increasing 15-PGDH expression, and reducing PG receptor mRNA levels. We believe that these actions contribute to suppress the proliferative stimulus provided by PGs in prostate cancer cells. Calcitriol treatment attenuated PG-mediated functional responses, including the stimulation of prostate cancer cell growth, the secretion of PGs to the conditioned media of PCa cells treated with calcitriol and blocking the up-regulation of PG target genes. The combination of calcitriol with nonsteroidal anti-inflammatory drugs (NSAIDs), known inhibitors of the enzymatic activity of COX-2, synergistically acted to achieve significant prostate cancer cell growth inhibition at ≃2 to 10 times lower concentrations of the drugs than when used alone. The regulation of PG metabolism and biological actions constitutes an additional novel pathway of calcitriol action mediating its anti-proliferative effects in prostate cells. We propose that a combination of calcitriol and a nonselective NSAID, such as naproxen, might be a useful therapeutic and/or chemo-preventive strategy in prostate cancer, as it would achieve greater efficacy and allow the use of lower concentrations of both drugs, thereby reducing their toxic side effects. The action of calcitriol at the genomic level to suppress COX-2 gene expression will decrease the levels of COX-2 protein and allow the use of lower the concentrations of NSAIDs needed to inhibit COX-2 enzyme activity. The combination approach is an attractive therapeutic strategy in the treatment of PCa and can be translated to clinical trials.

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APPENDIX I

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Jacqueline Moreno	Postdoctoral fellow

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Center for Research and Advanced Studies, Mexico City. MEXICO	M.S.	1998	Cell Biology
Center for Research and Advanced Studies, Mexico City. MEXICO	Ph.D.	2002	Cell Biology

Doctoral Research. July 1998- October 2002. Department of Physiology, Biophysics and Neurosciences, Center for Research and Advanced Studies. Research Advisor Dr. Marcelino Cereijido.

Molecular and cell biology of the epithelial transporting phenotype.

- Study of the sorting signals and mechanisms involved in membrane protein targeting.
- Analysis of protein-protein interaction motifs and their involvement in determining protein stability and retention.
- Role of tight junction associated protein phosphorylation on epithelial cell functional integrity.

Research Experience

Postdoctoral Associate. October 2002-June 2003, Center for Research and Advanced Studies. Study of the role of tight junction proteins and Na,K-ATPase _-subunit on epithelial transport phenotype genesis and maintenance.

Postdoctoral Fellow. November 2003- . Stanford University. Analysis of the mechanisms of 1_,25-DihydroxyVitamin-D3.

Scholarships

1993-1999. Fellow of the National Science and Technology Council (CONACyT, Mexico).

2004- Postdoctoral Traineeship Award. Department of Defense Prostate Cancer Research Program (PC04120).

Publications and Presentations

- Avila-Flores, A., Rendon-Huerta, E., **Moreno, J.**, Islas, S., Betanzos, A., Robles-Flores, M., Gonzalez-Mariscal, L. 2001. Tight-junction protein zonula occludens 2 is a target of phosphorylation by protein kinase C. *Biochem. J.* **360**: 295-304
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- Feldman, D., **Moreno, J.**, Krishnan, A.V. 2004. Pathways mediating the growth inhibitory actions of vitamin D in prostate cancer. Abstract in the Workshop on Cancer Chemoprevention & Cancer Treatment: Is there a role for vitamin D, 1α,25(OH)$_2$-vitamin D$_3$ or new analogs (deltanoids)?. NIH, Bethesda, MD.
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- **Moreno, J.**, Krishnan, A.V., Feldman, D. 2005. Molecular mechanisms mediating the anti-proliferative effects of Vitamin D in prostate cancer. *J. Steroid Biochem. Mol. Biol.*
- **Moreno, J.**, Krishnan, A.V., Swami, S., Nonn, L., Peehl, D.M., Feldman, D. 2005. Regulation of prostaglandin metabolism by calcitriol attenuates growth stimulation in prostate cancer cells. *Cancer Res.* **65**: 7917-7925.

Scientific Meetings:

- Feldman, D., Moreno, J., Krishnan A.V. Pathways mediating the growth inhibitory actions of Vitamin D in prostate cancer. 10th Prouts Neck Meeting on Prostate Cancer, Prouts Neck, Maine October 2004,
- Feldman, D., Moreno, J., Krishnan A.V. Pathways mediating the growth inhibitory actions of Vitamin D in prostate cancer. Vitamin D symposium: Cancer Chemoprevention & Cancer Treatment: Is there a role for vitamin D, 1α,25(OH)[**26**]2-vitamin D3 or new analogs (deltanoids)" November, 2004, Bethesda, Maryland.
- Moreno, J., Krishnan, A.V., Feldman, D. Pathways mediating the growth inhibitory actions of Vitamin D in prostate cancer. Abstracts of the 2nd Int. Symposium on Vitamin D Analogs in Cancer Prevention and Therapy. May 2005, Lübeck, Germany. *Anticancer Res.* 25. 2290.
- Moreno J., Krishnan AV, Feldman D. Regulation of prostaglandin metabolism by calcitriol: Potential role in the treatment of prostate cancer. Endocrine Society's 87th Annual Meeting, in San Diego California, June 2005.

APPENDIX II

Publications:

- *Exp. Cell Res.* **297**: 247-258.
- *Mol. Biol. Cell.* **16**: 1071-1081.
- *J. Steroid Biochem. Mol. Biol.*
- *Cancer Res.* **65**: 7917-7925.

Scientific Meetings:

- 10th Prouts Neck Meeting on Prostate Cancer, Prouts Neck, Maine October 2004,
- Abstracts of the 2nd Int. Symposium on Vitamin D Analogs in Cancer Prevention and Therapy. May 2005, Lübeck, Germany. *Anticancer Res.* 25. 2290.
- Endocrine Society's 87th Annual Meeting, in San Diego California, June 2005.

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Characterization of the tight junction protein ZO-2 localized at the nucleus of epithelial cells

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Abstract

ZO-2 is a MAGUK protein that in confluent epithelial sheets localizes at tight junctions (TJ) whereas in sparse cultures accumulates in clusters at the nucleus. Here, we have characterized several nuclear properties of ZO-2. We observe that ZO-2 is present in the nuclear matrix and co-immunoprecipitates with lamin B_1 and actin from the nuclei of sparse cultures. We show that ZO-2 presents several NLS at its amino region, that when deleted, diminish the nuclear import of the ZO-2 amino segment and impair the ability of the region to regulate the transcriptional activity of promoters controlled by AP-1. Several RS repeats are detected in the ZO-2 amino segment, however, their deletion does not preclude the display of a speckled nuclear pattern. ZO-2 displays two putative NES. However, only the second one appears to be functional, as when conjugated to ovalbumin (OV), it is able to translocate this protein from the nucleus to the cytoplasm in a leptomycin B-sensitive way.

Keywords: Tight junctions; ZO-2; MAGUK; Nuclear matrix; NLS; NES; PKC

Introduction

In multicellular organisms, the cellular sheets of epithelia and endothelia constitute the frontier between the internal milieu and the fluids contained within the different body compartments. Epithelial cells display tight junctions (TJ) at the apex of their lateral membranes. These structures regulate the passage of ions and molecules through the paracellular pathway and maintain epithelial polarity by blocking the free diffusion in the plasma membrane plane, of lipids and proteins.

In recent years, a wide array of cortical and integral proteins have been identified at the TJ [1]. Among the former, ZO proteins have been profusely studied. These proteins belong to the MAGUK family, characterized for presenting several conserved domains including three PDZ, one SH3 and a GK module [2], elements indicating that such proteins might serve multiple purposes. ZO-2 is a 160-kDa molecule originally identified as a TJ protein due to its co-immunoprecipitation with ZO-1 [3]. ZO-2 interacts as well with other tight and adherens junction proteins, such as occludin [4], claudins [5], cingulin [6] and α-catenin [4].

It has long been known that the TJ is anatomically and functionally associated to the cytoskeleton, and more recently, binding of actin filaments to ZO proteins has been observed [7].

The subcellular localization of ZO-2 is strongly sensitive to the state of cell–cell contact displayed by the monolayer. Thus, in sparse epithelial cultures, ZO-2 accumulates in clusters at the nucleus, where it partially co-localizes with splicing factor SC-35 [8]. Shuttling of ZO-2 between the TJ region and the nucleus might be achieved by the presence of putative nuclear localization [9] and exportation signals (NLS and NES) [8] on its sequence. The functional significance of the nuclear distribution of ZO-2 still remains unclear; therefore, characterizing the properties of nuclear ZO-2 is crucial for elucidating its physiological role. Here, we have studied ZO-2 association to nuclear matrix proteins and the role that NLS and NES plays on its shuttling.

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Material and methods

Cell culture

Starter epithelial Madin Darby canine kidney (MDCK) cultures were obtained from the American Type Culture Collection (MDCK, CCL34). Cells between the 60th and 90th passage were grown at 36.5°C in disposable plastic bottles (Costar 3151, Cambridge, MA) with a 95% air, 5% CO_2 atmosphere (Stericult 200, Forma Scientific, Marrieta, OH) and 20 ml of Dulbecco's modified Eagle's basal medium (DMEM; D1152 Sigma Co., St. Louis, MO) with penicillin (100 IU/ml; Eli Lilly, México) and 10% iron-supplemented certified calf serum (Gibco BRL, 10371-029, Grand Island, NY). Cells were harvested with trypsin–EDTA (In Vitro, México) and plated either sparse (1×10^5 cells/cm^2) or at confluency (3×10^5 cells/cm^2).

Elaboration of total cellular extracts and nuclear matrix preparations

MDCK cells were lysed under gentle rotation for 15 min at 4°C with RIPA buffer (40 mM Tris–HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 1 mM PMSF) containing the protease inhibitor cocktail Complete™. The lysate was then sonicated three times for 30 s each in a high-intensity ultrasonic processor (Vibra cell, Sonics and Materials Inc., Danbury, CT).

For the Western blot experiments, nuclear matrix was prepared according to the protocol described by Cook et al. [10] with slight modifications introduced by Aranda-Anzaldo and Dent [11]. Briefly, cells derived from sparse cultures were lysed in a buffer containing 2.6 M NaCl, 1.3 mM EDTA, 2.6 mM Tris and 0.6% of Triton X-100, pH 8.0. After 20 min at room temperature, 200 μl of this lysate containing 1×10^6 cells were applied on top of a sucrose gradient (15–30%; 600–200 μl; pH 8.0) containing 2 M NaCl, 10 mM Tris and 1 mM EDTA. The gradients were spun at 4°C on a Sorvall Biofuge Fresco centrifuge at 10,000 rpm. The nucleoids present as a white aggregate at the gradient interphase between the two layers of sucrose were collected with a Pasteur pipette, intensively washed with PBS and digested with DNAse I (catalogue no. E2215Y; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) in a buffer containing 10 mM $MgCl_2$, 0.2 mM 2-mercaptoethanol and 50 mM Tris, pH 7.2, for 10 min at 37°C. The DNA digestion was stopped with a solution of 200 mM EDTA and 10 mM Tris. After 10 min on ice, the samples were spun for 15 min at 4°C on a Sorvall Biofuge Fresco centrifuge at 13,000 rpm. The pellet obtained was resuspended in RIPA buffer with the protease inhibitor cocktail Complete™.

For the generation of the extracted monolayers to be observed by immunofluorescence, we employed a protocol designed by Fey et al. [12] and slightly modified by Padros et al. [13] and Vindrola et al. [14]. Briefly sparse monolayers grown on glass coverslips were rinsed with phosphate-buffered saline (PBS, pH 7.4) and fixed in 2% *p*-formaldehyde for 30 min at 4°C. The cells were then extracted in cytoskeletal buffer (CSK; 100 mM NaCl, 300 mM sucrose, 10 mM PIPES, pH 6.8, 3 mM $MgCl_2$, 0.5% Triton X-100, 1.2 mM phenylmethylsulfonyl fluoride and 1 mM EGTA) for 10 min a 4°C. The resulting soluble fraction was removed. Cells were then further extracted with RSB buffer (250 mM ammonium sulfate, 10 mM NaCl, 10 mM Tris, pH 7.4, 3 mM $MgCl_2$, 0.5% Triton X-100, 1.2 mM phenylmethylsulfonyl fluoride and 1 mM EGTA) to remove the salt labile cytoskeleton. The chromatin fraction was digested employing a buffer identical to the CSK buffer with the exception that it contained 50 mM NaCl. To this buffer we added 250 units/ml of bovine pancreatic DNAse I and RNAse (Catalogue no. 5305-888777 5 Prime-3 Prime Inc., Boulder CO, USA) and digestion proceeded for 20 min at room temperature. Chromatin-associated proteins were released by the addition of ammonium sulfate to a final concentration of 250 mM, and incubation continued for 5 min at room temperature. The chromatin fraction was removed from the monolayers, leaving the nuclear matrix-intermediate filaments (NM-IF) fraction.

Protein blotting

In total extracts and nuclear matrix preparations, proteins were quantified and the samples were diluted (1:1) in treatment buffer (125 mM Tris–Cl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, pH 6.8), run in 8% polyacrylamide gels and transferred to PVDF membranes (Hybond RPN303F; Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK).

Blotting was performed with polyclonal antibodies against ZO-2 (catalogue no. 71-1400, dilution 1:500; Zymed laboratories, San Francisco, CA, USA), ZO-1 (catalogue no. 61-7300, dilution 1:500; Zymed laboratories), His-probe (catalogue no. sc-803, dilution 1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and mouse monoclonals against lamin B_1 (Zymed Zs33-2000; dilution 1:500), β Na^+-K^+-ATPase (a generous gift of Dr. Marcelino Cereijido, CINVESTAV, México), Histone 4 (catalogue no. 06-753, dilution 1:500; Upstate Biotechnology, Lake Placid, NY, USA) and actin (a generous gift of Dr. José Manuel Hernández, CINVESTAV, México; dilution 1:5). Peroxidase-conjugated goat IgG against rabbit IgG or against mouse IgG (catalogue nos. 62-6120 and 62-6520, respectively; dilution 1:2000; Zymed Laboratories) were used as secondary antibodies, followed by a chemiluminescence detection system (ECL+PLUS, RPN 2132; Amersham Pharmacia Biotech). Alternatively, goat anti-rabbit or anti-mouse antibodies coupled to alkaline phosphatase (catalogue nos. 170-6518 and 170-6520, respectively; Bio-Rad Laboratories) were used and a 5-bromo-4-chloroindol-3-yl phosphate/Nitro Blue Tetrazolium colorimetric detection kit

(catalogue no. 170-6432; Bio-Rad) was subsequently employed.

Immunofluorescence

MDCK monolayers cultured on glass coverslips were extensively washed in PBS and fixed in 2% *p*-formaldehyde for 30 min at 4°C. Some of these monolayers were then processed to obtain the NM-IF fractions, whereas others were immediately processed for immunofluorescence. The latter protocol started with a 30-min blockade with 1% BSA Ig free (Research Organics catalogue no. 1331-a). The monolayers were then incubated overnight at 4°C with a rabbit polyclonal antibody against ZO-2 (diluted 1:100 in 1% Ig-free BSA) or monoclonals Anti-Xpress (catalogue no. R910-25, dilution 1:500; Invitrogen Life Technologies, Carlsbad, CA, USA) or anti-HA (catalogue no. sc-7392, dilution 1:100; Santa Cruz Biotechnology Inc.). The coverslips with the monolayers were then washed five times with PBS and incubated for 1 h at room temperature with a secondary antibody (FITC-conjugated goat anti-rabbit, catalogue no. 65-6111; diluted 1:100; Zymed). After being washed three times, the monolayers on the glass coverslips were mounted with the antifade reagent Vectashield (Vector Laboratories Inc., Burlingame, CA). The fluorescence of the monolayers was examined using a confocal microscope (MRC-600, Bio-Rad) with a krypton–argon laser.

Immunoprecipitation of ZO-2 from the nuclei of MDCK cells

Nuclei derived from sparse MDCK cells were isolated according to a procedure previously described [15]. Briefly, the monolayers were treated with buffer A: 10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40; vortexed for 30 s, and centrifuged at 1000 × *g* for 5 min. The pellet was treated again with buffer A, and the procedure was repeated three times. The efficiency of the cellular lysis was checked by adding Hoechst (data not shown). For immunoprecipitation, the nuclear pellet or a total cell extract was lysed under gentle rotation for 15 min at 4°C with RIPA buffer (40 mM Tris–HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 1 mM PMSF) diluted 1:1 with HO buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100) containing the protease inhibitor cocktail Complete™. The extract obtained was centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatants were collected and cleared with *Staphylococcus aureus* cells conjugated with IgG goat antibodies to IgG rabbit antibodies (Tachisorb cells, 100 μl/24 × 10^6 cultured cells, catalogue no. 575544; Calbiochem, La Jolla, CA, USA) for 1 h at 4°C. After centrifugation (10 min at 1300 × g), supernatants were incubated overnight at 4°C, with the polyclonal ZO-2 antibody (1 μg/24 × 10^6 cultured cells) under continuous rotation. Tachisorb cells were added and incubated 1 h at 4°C. Immunoprecipitates were washed twice with HO buffer followed by three washes with 50 mM Tris–HCl (pH 7.4). The pellets were boiled 10 min in sample buffer and electrophoresed onto 12% polyacrylamide-SDS gels.

ZO-2 fusion proteins

Here, we employed two ZO-2 GST fusion proteins, whose construction was previously described by us [16]. The first named 3PSG included the third PDZ domain, the SH3 region and the GK module (residues 400–874 of canine ZO-2 [17]); the second called AP covered the acidic and proline-rich domains of ZO-2 (residues 878–1179 of canine ZO-2 [17]).

Pull-down assays

Pull-down assays were performed employing 1 mg of a nuclear lysate isolated from rat kidneys and 25 μg of 3PSG-GST or AP-GST fusion proteins as previously described [18].

Expression plasmids, transfection and CAT assays

Full-length canine ZO-2 introduced into the CMV expression plasmid GW1 was kindly provided by Ronald Javier (Baylor College of Medicine, Houston, TX). The middle (3PSG; 1595–3019 nt) and carboxyl-terminal (AP; 3029–3923 nt) segments of canine ZO-2 were introduced into pcDNA4/HisMax by us as previously reported [18]. The amino-terminal (398–2165 nt) segment of canine ZO-2, containing PDZ1, PDZ2 and PDZ3, was amplified by RT-PCR using elongase (Cat. No. Y02370; Life Technologies, Grand Island, NY) and sequentially introduced into the *Eco*RV site found in the multiple cloning site of pBluescript II KS (+) (catalogue no. 212207; Stratagene, La Jolla, CA). To generate the amino-ZO-2 ΔNLS segment, we restricted the amino-ZO-2/pBluescript construct with the *Hpa*I and *Pfl*MI enzymes producing the deletion of nucleotides 592–1486 (see scheme in Fig. 4). The amino-ZO-2 ΔPDZ2,PDZ3 segment was generated by cutting the amino-ZO-2/pBluescript construct with *Xho*I and *Eco*RI, producing the elimination of nucleotides 957–2165. The amino segments of ZO-2 were then introduced into the pcDNA4/HisMaxB vector (Cat. No V864-20; Life Technologies). The Plasmids were purified employing the QIAGEN plasmid purification kit (Cat. No. 12142; Chatsworth, CA) and verified by restriction and complete sequence analyses.

The reporter gene TRE-CAT was kindly donated by Michael Gredes from Dr. Yuspa laboratory at NIH. TRE-CAT contains the structural gene for chloramphenicol acetyltransferase (CAT) under the control of the Herpes virus thymidine kinase (HSV-TK) promoter and five SV40 AP-1 sites cloned upstream [19].

Transfections on MDCK cells were carried out with Lipofectamine 2000 reagent (Cat. No. 11668-019; Life Technologies) as recommended by the manufacturer.

CAT assays were performed as previously described by us [18] in sparse cells to obtain a higher expression of the transfected construct in the nucleus.

Microinjection of rhodaminated albumin and ZO-2 NES peptides conjugated to ovalbumin

Peptides corresponding to the NES-1 ($_{360}$LQLVVLRD-SK$_{370}$) and NES-2 ($_{728}$LEKLANELPDL$_{738}$) sequences of ZO-2 were conjugated to ovalbumin (OV) by Research Genetics Invitrogen Corporation. The coupling ratio of both conjugations was of one NES peptides per OV molecule. Where indicated, cells were incubated before microinjection, in media containing 50 nM leptomycin B, kindly donated by Dr. M. Yoshida from the Department of Biotechnology at Tokyo University, Japan.

Microinjection was performed using an IM 300 apparatus (Narishige, Tokyo, Japan) with borosilicate pipettes that have attained a resistance of 1 MΩ with a horizontal Brown/Flaming puller (Sutter P97, Novato, CA, USA), and employing a piezoelectric micromanipulator (Burleigh, PCS500, New York, USA). The pipettes were filled with 1 μl of a solution containing the samples [0.5 mg/ml rhodaminated albumin (Cat. No. A-847; Molecular Probes, Eugene, OR, USA) plus 0.4 mg/ml of either ZO-2 NES-1 or NES-2 conjugated to ovalbumin] diluted in PBS. The microinjection was performed on the nuclei of sparse cells plated 24 h before on glass coverslips and was verified by the epifluorescence observation of the injected rhodaminated albumin in an inverted microscope (Nikon Diaphot 200, Tokyo, Japan). Thirty minutes after the nuclear injection, the monolayers were fixed with 4% *p*-formaldehyde and processed for immunofluorescence employing a polyclonal rabbit anti-ovalbumin antibody generated and generously provided by Dr. Vianney Ortiz Navarrete (CINVESTAV, México), and the secondary fluoresceinated anti-rabbit antibody described above.

Results

Nuclear ZO-2 is associated to the nuclear matrix

To determine if ZO-2 found at the nucleus of sparse epithelial cells is associated to the nuclear matrix, we generated from MDCK epithelial cells a total extract as well as a nuclear matrix preparation. Fig. 1A illustrates how the nuclear matrix preparation is devoid of the β subunit of the Na^+-K^+-ATPase, employed here as a plasma membrane

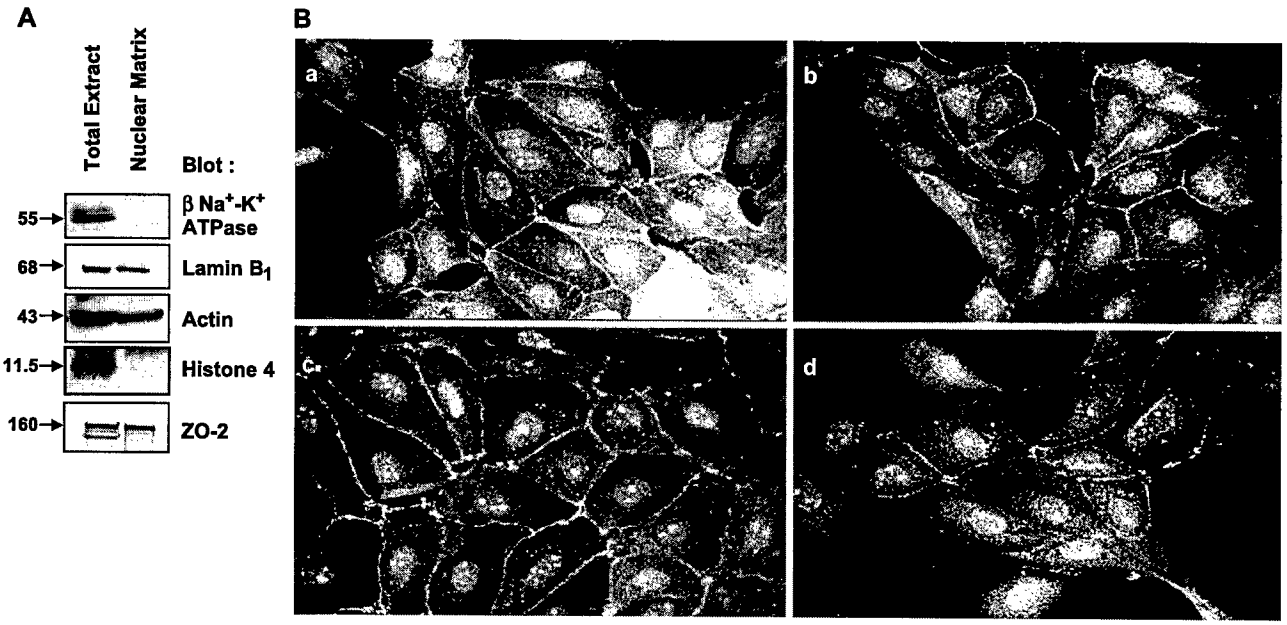


Fig. 1. The TJ protein ZO-2 found at the nucleus is associated to the nuclear matrix. (A) A total extract and a nuclear matrix preparation derived from sparse MDCK cells was separated by SDS-PAGE, transferred to PVDF membranes and blotted with specific antibodies against the plasma membrane protein β subunit of the Na^+-K^+-ATPase; the nuclear matrix proteins lamin B_1 and actin; the soluble nuclear protein histone 4 and the TJ protein ZO-2. Observe how at the nuclear matrix lamin B_1, actin and ZO-2 can be clearly detected, although the preparation is devoid of β Na^+-K^+-ATPase and histone 4. In this and the following figures, the numbers aside of the arrows indicate the molecular weight in kilodaltons of the blotted proteins. (B) Immunofluorescence detection of ZO-2 in the nuclear matrix. *p*-Formaldehyde-fixed monolayers of sparse MDCK cells were rinsed twice with PBS (a) and then sequentially extracted with buffers to release the soluble proteins (b), the salt labile cytoskeleton (c) and the chromatin associated proteins (d). Observe how ZO-2 remains detectable at the nucleus and cellular border throughout the whole extraction procedure.

marker [20], and of the soluble nuclear protein Histone 4, although both proteins can be detected in the total cellular extract. The nuclear matrix preparation instead displays the presence of lamin B_1 and actin, which are characteristic components of the nuclear matrix [21,22]. ZO-2 was detected in both the total cellular extract and the nuclear matrix, suggesting the association of this protein to the nuclear matrix.

To explore this possibility, we performed on sparse MDCK monolayers a sequential extraction (Fig. 1B) of soluble proteins with a 0.5% Triton X-100 buffer (b) of the salt labile cytoskeleton employing an ammonium sulfate containing buffer (c) and of chromatin-associated proteins by submitting the monolayers to a treatment with DNAse and RNAse (d). The results depicted in Fig. 1B illustrate how, despite the treatment to which the monolayers are subjected, ZO-2 remains as in control monolayers (a) located at both the cell–cell borders and the nucleus, thus reinforcing the idea that nuclear ZO-2 is in vivo strongly associated to the nuclear matrix.

To further test the association of ZO-2 to nuclear insoluble proteins, we immunoprecipitated ZO-2 from a nuclear preparation derived from sparse MDCK monolayers. As Fig. 2 shows, nuclear matrix proteins, actin and lamin B_1 can be detected in the ZO-2 immunoprecipitate. The association of ZO-2 with lamin B_1 is a novel observation, therefore, we proceeded to study if different segments of ZO-2 maintained this interaction. We started by transfecting MDCK cells with a poly-histidine-tagged construct named amino ZO-2 (Fig. 3), which comprises the three PDZ domains and the NLS of the protein. Fig. 4A shows how

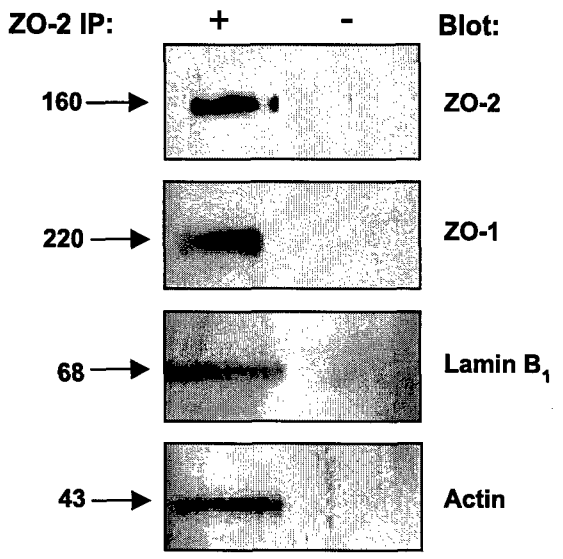


Fig. 2. Lamin B_1, actin and ZO-1 co-immunoprecipitate with ZO-2 obtained from the nuclei of sparse monolayers. Immunoprecipitates (IP) performed from the nuclei of sparse monolayers with antibodies against ZO-2 or with pre-immune serum (–) were run on SDS-PAGE, transferred to PVDF membranes and blotted with antibodies against lamin B_1, actin, ZO-1 and ZO-2.

lamin B_1 does not co-immunoprecipitate with the amino ZO-2 segment, although it is clearly detected in the total cellular extract (TE). Since the remainder segments of ZO-2 do not contain NLS, which could facilitate the entrance of the proteins to the nucleus, we continued our study performing pull-down assays from a kidney nuclear lysate using GST fusion proteins corresponding to the middle (3PSG) and carboxyl-terminal (AP) domains of ZO-2 (Fig. 3). As Fig. 4B shows, lamin B_1 is conspicuously present in the nuclear lysate (NL) and the AP pull-down, and to a lesser extent in the assay performed with the 3PSG fusion protein. These results hence indicate that lamin B_1 interacts with the carboxyl half of ZO-2.

As stated in the Introduction, ZO-2 was originally identified as a protein that co-immunoprecipitates with ZO-1. Since the latter protein has also been reported to shuttle to the nucleus upon conditions of low cell–cell interaction [23], we tested if in the ZO-2 nuclear immunoprecipitate ZO-1 could be detected. Fig. 2 shows the conspicuous presence of ZO-1 in the ZO-2 nuclear immunoprecipitate, thus suggesting that as in the plasma membrane, at the nucleus, both ZO proteins form part of the same complex.

The amino segment of ZO-2 presents several NLS and RS repeats whose deletion decreases, but does not inhibit, the nuclear import of the molecule

In a previous work, we demonstrated the presence of NLS in several MAGUK proteins of the TJ including ZO-2 [9]. For the latter, we initially described a bipartite signal (aa 83–99 in Fig. 3) located between the last portion of the first PDZ repeat and the first residues of linker-1, the region between PDZ1 and 2. Recently however, it has been shown that deletion of this sequence does not preclude the entrance to the nucleus of an amino ZO-2 segment, prompting to speculate the presence of one or more additional NLS domains in the N-terminal region of ZO-2 [24]. We thus performed a search for new putative NLS present in ZO-2. Fig. 3 illustrates the presence of previously undescribed NLS present in the sequence of ZO-2: (a) A bipartite NLS (aa 246–262, double underlined) that as the first described ZO-2 NLS (aa 83–99, double underlined) follows the pattern of two basic residues (K or R), a 10-residue spacer and another basic region with three basic amino acids (K or R) out of five residues. (b) Three monopartite NLS, composed of three basic amino acids (K or R) and either H or P, one located within the basic region (aa 185–188, underlined) and the other two overlapping the terminal portion of the first bipartite NLS (brackets). (c) We further found that the basic domain of ZO-2 (Fig. 3, +), contained within the first linker of the molecule, has numerous arginine/serine (RS) dipeptide repeats (bold). The latter have been described in transcriptional activators [25,26], as well as in a large family of eukaryotic pre-mRNA splicing factors named SR proteins [27]. The RS-rich domain is necessary and sufficient for targeting proteins to nuclear speckles and

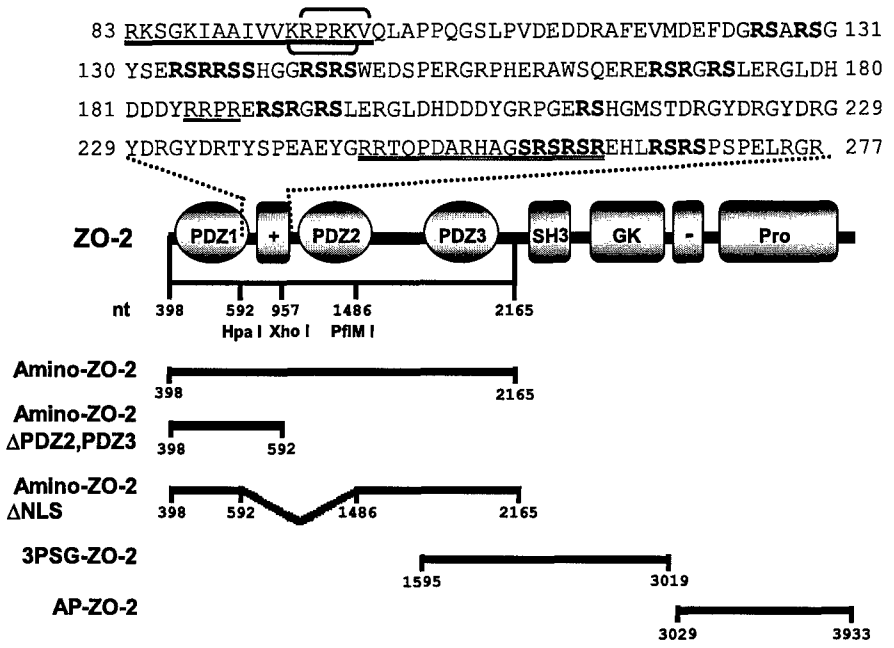


Fig. 3. Schematic representation of ZO-2 showing the NLS and RS motifs located within the amino-terminal portion of the molecule and the different ZO-2 constructs. The predicted amino acid sequence reveals the presence of two bipartite NLS (double underlined residues) and three monopartite NLS, one located within the basic region (underlined), whereas the other two are contained within the terminal portion of the first bipartite NLS (brackets). The dipeptide repeats of arginine and serine (RS) are shown in bold. The lower diagrams illustrate the amino-ZO-2 segment and its deletion fragments: the amino-ZO2 ΔPDZ2,PDZ3 and the amino-ZO-2 ΔNLS, as well as the middle (3PSG-ZO-2) and carboxyl-terminal (AP-ZO-2) segments of ZO-2.

constitutes the only known sequence that is shared by all the splicing factors that localize to speckles [28]. Speckles were originally described as nuclear storage sites for splicing factors but are now perceived as active places of coordinate transcription and splicing [29]. Therefore, the observation that in sparse epithelial cells ZO-2 is found at nuclear speckles [8] suggested that this protein could play a role in nuclear transcription, a situation that has recently been

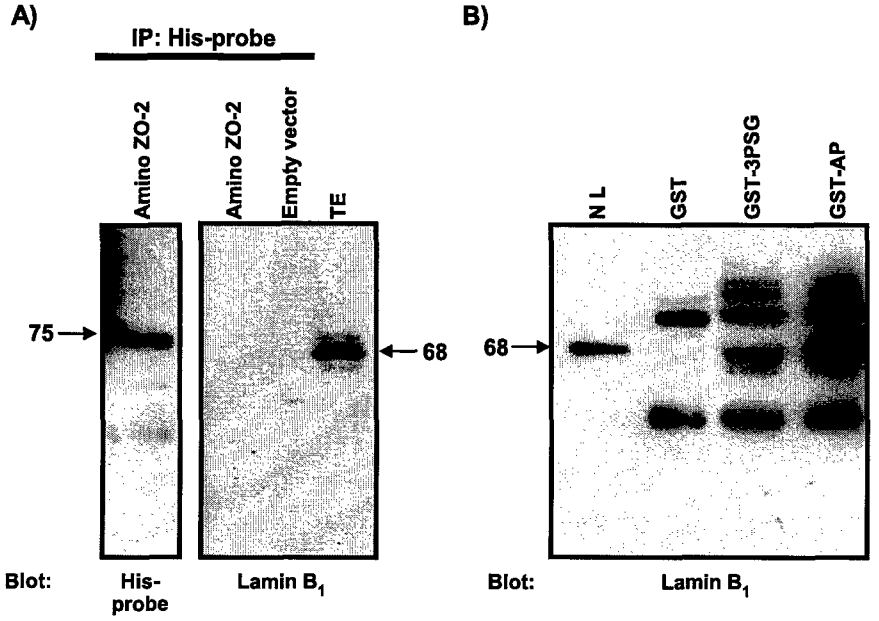


Fig. 4. The middle and carboxyl-terminal domains of ZO-2 interact with lamin B_1. (A) Lamin B_1 does not co-immunoprecipitate with the transfected amino ZO-2 segment. MDCK cells transfected with the amino ZO-2 construct or the empty vector were immunoprecipitated with antibodies against the His probe. The IP was blotted with antibodies against His probe or lamin B_1. TE: total cellular extract. (B) In a pull-down assay, ZO-2 fusion proteins 3PSG and AP interact with lamin B_1. GST, GST-3PSG and GST-AP interacted with a nuclear lysate (NL) obtained from rat kidney. After an extensive wash and a SDS-PAGE, the presence of lamin B_1 was revealed with a specific antibody.

demonstrated by us, for reporter genes regulated by AP-1-controlled promoters [18].

To determine if the NLS and RS repeats of ZO-2 were capable of directing ZO-2 to the nucleus, we proceeded to transfect MDCK cells with the following ZO-2 amino constructs containing both Xpress and polyhistidine tags (Fig. 3): (a) the complete amino segment; (b) a construction named amino-ZO-2 ΔPDZ2,PDZ3, containing the first PDZ domain and the first bipartite NLS of ZO-2 (nt 398–957); and (c) the amino-ZO-2 ΔNLS construct lacking all the NLS and RS repeats found within the first linker of the molecule (ΔNLS looses nt 593–1486). We then analyzed by immunofluorescence with an anti-Xpress antibody the cells displaying predominantly nuclear, a mixed nuclear-cytosolic or an exclusive cytosolic localization of the amino ZO-2 segments (Fig. 5A, inserts). The histograms of Fig. 5A show that the number of nuclear-stained cells was significantly higher in sparse than in confluent monolayers. In contrast, in sparse cultures, a very low proportion of cells were found to display a sole cytosolic localization of the amino ZO-2 fragments. In summary, these results are in agreement with our previous observation concerning the inverse relationship between the movement of ZO-2 to the nucleus and the degree of cell–cell contact in the monolayer [8].

Upon comparing the distribution of the different amino fragments in the transfected cells, we observe that the absence of the monopartite and the second bipartite NLS of ZO-2 in the amino-ZO-2 ΔPDZ2,PDZ3 construct decreases the nuclear localization of the protein. Furthermore, when all the NLS and RS repeats of the molecule are eliminated in the amino ZO-2 ΔNLS, almost no nuclear localization is detected. Thus, suggesting that all the NLS of the molecule contribute to its nuclear import. In agreement with these results, the sole cytosolic distribution of the constructs increases as the molecules loose their NLS. Now, with regards to the nuclear-cytosolic distribution, it is clear that it is more abundant in sparse than in confluent monolayers, again reflecting the importance of the extent of cell–cell contact for ZO-2 distribution. The fact that even in the transfection with the ΔNLS construct, lacking all the NLS and RS repeats of the molecule, a strong nuclear-cytosolic distribution is observed (75% in sparse cultures), suggests the possibility that ZO-2 enters the nucleus by a piggyback mechanism.

When we analyzed the staining pattern of the different ZO-2 amino constructs, we observed that even with the amino-ZO-2 ΔNLS segment, a speckled distribution is displayed at the nucleus (Fig. 5B). Both the entrance to the nucleus and the maintenance of the speckled pattern could possibly be explained by the recent observation that the DNA binding protein SAF-B (scaffold attachment factor-B), which contains NLS and distributes in nuclear speckles [30,31], interacts with the first PDZ domain of ZO-2 [24]. Interestingly, in another study where MDCK cells were transfected with an amino ZO-2 construct, lacking the first PDZ but containing the RS repeats, the protein appeared uniformly distributed in the nucleus and hardly any speckles were visible [24]. Together, these observations strongly suggest that for the ZO-2 amino segment to display a speckled distribution at the nucleus, the presence of an intact PDZ1 repeat is necessary.

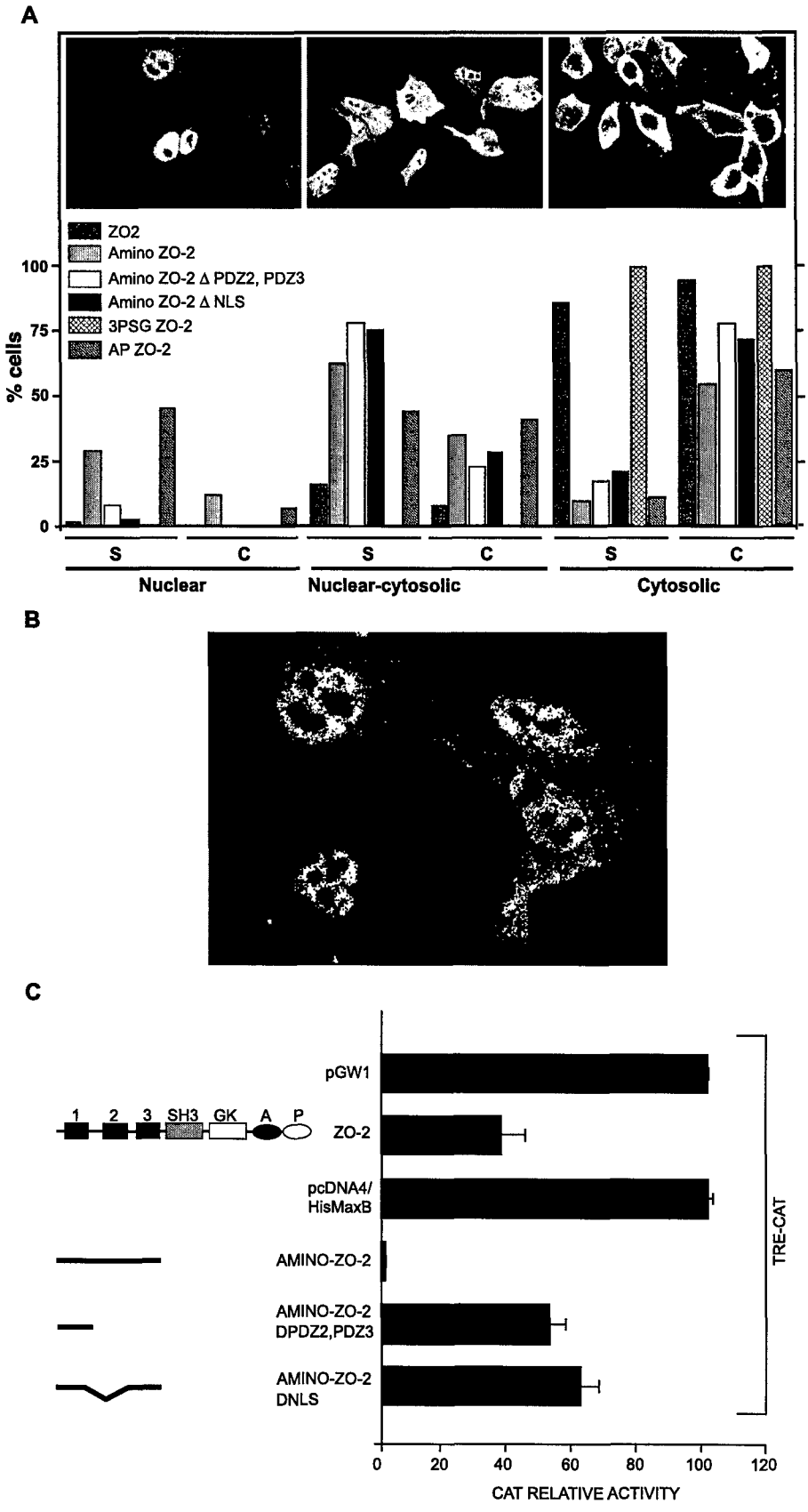
When epithelial cells are transfected with full-length ZO-2, the middle or the carboxyl-terminal domains of the molecule, only the latter segment is efficiently expressed at the nucleus

Next, we proceeded to analyze the subcellular distribution of transfected full-length ZO-2, as well as of the middle and carboxyl-terminal domains of the molecule. Fig. 5A shows how the percentage of cells displaying a nuclear and a nuclear-cytosolic distribution of full-length ZO-2 and of 3PSG is significantly lower than that observed with the amino ZO-2 transfection. This result could be due to the NES present in the GK domain of ZO-2 [8], which could promote the export of the molecule from the nucleus, and to the putative association of ZO-2 to occludin at the TJ, through its GK region. Although the latter interaction has not been demonstrated for ZO-2, it has been clearly established for ZO-1 [4,32,33]. Here, a clear observation of transfected full-length ZO-2 at the plasma membrane became difficult due to the high amount of this protein present in the cytosol.

In contrast, the percentage of cells displaying a nuclear and nuclear-cytosolic (N + NC) distribution of the AP domain of ZO-2 is somewhat similar to that observed with the amino-ZO-2 construct. Although the AP region does not posses any known NLS that could facilitate its entrance into the nucleus, it associates to transcription factors Jun, Fos and C/EBP, in pull-down and gel shift assays [18]. This interaction could hence allow, by a piggy-back mechanism, the nuclear import of the AP domain.

The amino constructs of ZO-2 are capable of inhibiting a reporter gene controlled by AP-1, albeit at different intensities

Recently, we demonstrated that ZO-2 is capable of modulating the transcriptional activity of promoters controlled by AP-1 [18]. When in that work we proceeded to determine which segment of ZO-2 was responsible for such an effect, we observed that the over-expression of the amino-ZO-2 ΔPDZ2,PDZ3 and the AP segments, but not of the 3PSG region, repressed the activity of the reporter gene CAT, controlled by AP-1 [18]. These effects correlate with the respective nuclear presence and absence of the AP and 3PSG segments, shown in Fig. 5A. Therefore, here we proceeded to study if the activity of CAT varied in accordance to the efficiency of the nuclear arrival of the different amino constructs. We performed co-transfections of MDCK cells with full-length ZO-2, the amino ZO-2 constructs or their respective empty vectors (pGW1 and pcDNA4/HisMaxB) and a CAT construction named TRE-CAT, which


A
ZO2
Amino ZO-2
Amino ZO-2 Δ PDZ2, PDZ3
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3PSG ZO-2
AP ZO-2
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pGW1
1 2 3 SH3 GK A P
ZO-2
pcDNA4/
HisMaxB
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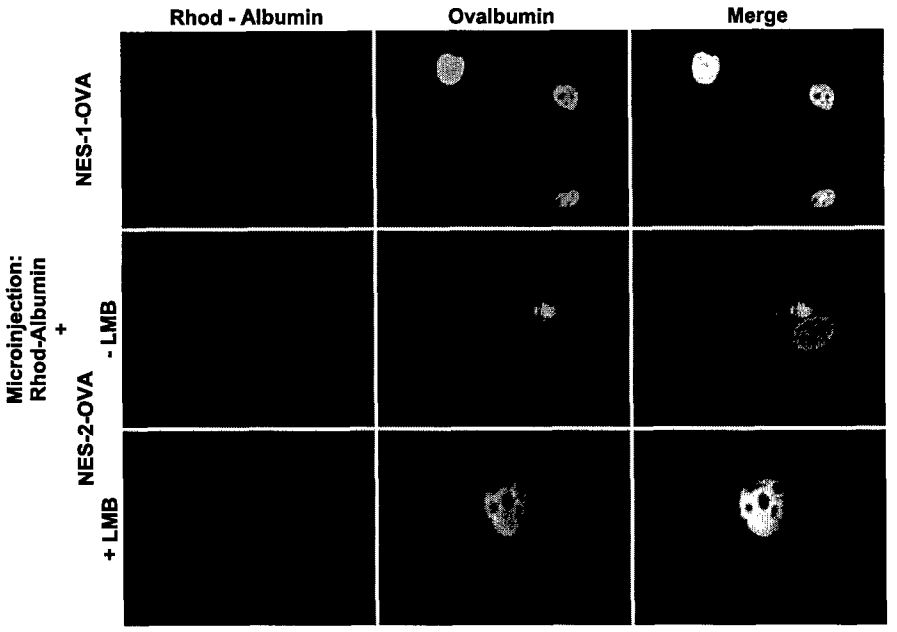

Fig. 6. A synthetic peptide corresponding to the second NES present in ZO-2 is able to direct ovalbumin from the nucleus to the cytoplasm in a leptomycin B-sensitive manner. A mixture of 0.5 mg/ml rhodaminated (rhod) albumin and 0.4 mg/ml ovalbumin (OVA) chemically conjugated to peptides corresponding to the putative NES-1 (upper panels) or NES-2 (middle and lower panels) present in ZO-2 was microinjected into the nuclei of MDCK cells. The cells in the lower panel were microinjected 1 h after treatment with 50 nM LMB. Cells were fixed 30 min after injection and stained with rabbit anti-OVA antibodies and secondary anti-rabbit fluoresceinated polyclonals. The first column illustrates the nuclear confinement of the injected rhodaminated albumin. In the second column, we observe how the NES-1-OVA remains at the nucleus, whereas NES-2-OVA exports the nucleus in a LMB-sensitive mode.

contains five AP-1 sites upstream of the HSV thymidine kinase promoter.

Fig. 5C shows how full-length ZO-2 significantly diminishes CAT activity relative to the empty vector, even if the percentage of transfected full-length ZO-2 expressed at the nucleus is relatively low (Fig. 5A). On analyzing the effect of the different amino constructs, we observe how CAT activity is almost completely abolished when the amino-ZO-2 segment, which proved to be most effective in its arrival to the nucleus (Fig. 5A), is transfected. In contrast, when the amino ZO-2 ΔNLS and the amino-ZO-2 ΔPDZ2, PDZ3 constructs were employed, a diminished inhibition of CAT activity was detected (Fig. 5C), although both of these ZO-2 segments still enter the nucleus efficiently. These results therefore suggest that the presence of the second PDZ domain, which is absent in both amino ΔNLS and amino-ΔPDZ2,PDZ3 constructs, does not alter significantly the entrance of the ZO-2 segments to the nucleus, but yet appears to increase the ability of the molecule to regulate AP-1-controlled promoters.

The second NES present in ZO-2 is able to translocate ovalbumin form the nucleus to the cytoplasm in a leptomycin B-sensitive way

Recently, we reported the presence of two NES in the sequence of ZO-2 [8]. To test whether such leucine-rich sequences can act as autonomous NES, the peptides corresponding to residues 361–370 and 728–738 of canine ZO-2 were synthesized and chemically conjugated to ovalbumin resulting, respectively, in NES-1-OVA and NES-2-OVA. When injected into the nucleus, NES-1-OVA remained within the nucleus together with the co-injected rhodaminated albumin (rhod-albumin) (Fig. 6, upper row). In contrast, when NES-2-OVA was injected, it was excluded from the nucleus almost completely within 30 min while the co-injected rhod-albumin remained in the nucleus (Fig. 6, middle row). The nuclear export of NES-2-OVA was sensitive to leptomycin B treatment (Fig. 6, lower row), an antifungal compound that exerts a potent and specific inhibition of the NES-dependent nuclear export of proteins,

Fig. 5. Upon transfection, full-length ZO-2, the amino constructs and AP are expressed at the nucleus and are capable of diminishing the transcriptional activity of promoters controlled by AP-1. (A) Sparse (S) and confluent (C) MDCK cells were transfected with full-length ZO-2 (▒ bars), the amino-ZO-2 (grey bars), the amino-ZO-2 ΔPDZ2,PDZ3 (empty bars), the amino-ZO-2 ΔNLS (black bars), the 3PSG ZO-2 (▒ bars) and the AP ZO-2 (▒ bars) constructs and treated with anti-tag antibodies to determine by immunofluorescence their subcellular localization. Quantification of the percentage of transfected MDCK cells showing predominant nuclear (left inset), mixed nuclear-cytoplasmic (middle inset) or exclusively cytoplasmic (right inset) localization is shown. At least 400 cells were analyzed for each condition. (B) Speckled distribution pattern observed at the nuclei of MDCK cells transfected with the amino-ZO-2 ΔNLS construct. (C) Reporter gene assay in which cells were transiently co-transfected with full-length ZO-2, the amino ZO-2 segments or their respective empty vectors pGW1 and pcDNA4/HisMaxB, plus a TRE-CAT construct that contains a promoter regulated by five AP-1 sites.

by preventing their association with the export receptor CRM1/exportin [34].

Discussion

In this study, we focused on the characteristics of nuclear ZO-2. We started our studies examining if ZO-2 is associated to proteins of the nuclear matrix. We found that the carboxyl-terminal half of ZO-2 is associated to lamin B_1. However, we cannot determine whether this interaction is direct or involves an intermediate protein until a recombinant lamin B_1 is available for the assays. The association of ZO-2 with lamin B_1 could speculatively suggest the participation of this MAGUK in chromatin organization since lamin B_1 is involved in this nuclear function [35]. We also detected interaction between ZO-2 and nuclear actin. Previous co-sedimentation studies had shown that ZO-2 interacts directly with F-actin in vitro, although it does not cross-link or bundle actin [7]. The carboxyl-terminal of ZO-2 has been proposed to function as the site of interaction with actin since an exogenously expressed C-terminal ZO-2 construct distributes along actin stress fibers in fibroblasts [4]. In confluent monolayers, a continuous apical actin belt appears in the same focal plane as that of proteins of the TJ, and treatment with cytochalasin B or the PKC agonist DiC8 induces F-actin and ZO proteins redistribution in similar patterns [7,36]. In these studies however, actin filaments were not detected in the nucleus. This apparent discrepancy resides in the fact that cytoskeletal studies have generally employed phalloidin staining of actin, and this compound binds only to actin polymers. Instead, actin at the nucleus has been proposed to exist either as monomers and short oligomers, or as long filaments coated with proteins that obstruct the phalloidin-binding pocket. Many reports have indicated the presence of actin in the nucleus and provide evidence for the role of actin in nuclear processes such as chromatin remodeling and RNA splicing (for review, see Ref. [37]). Therefore, the association of nuclear ZO-2 to actin could speculatively suggests the participation of ZO-2 in such processes. In this respect, we previously found that ZO-2 co-localizes with splicing factor SC-35 at spliceosomes, and several splicing factors have been found to associate to the nuclear matrix [38–40]. Furthermore, the cytoskeletal protein 4.1 that has been shown to co-immunoprecipitate with ZO-2 [41], is also present at the nucleus where it co-localizes with SC-35 [42] and participates in the splicing activity of different pre mRNA substrates [42].

The ZO-2 amino constructs here employed, including the amino mutant lacking all the NLS and RS repeats, were effectively expressed at the nucleus of sparse MDCK cells. However, a more efficient arrival to the nucleus was detected when all the NLS of the molecule were present. These observations suggest that for entering the nucleus, the ZO-2 amino segments might rely on their NLS as well as on their association to other proteins that are constantly traveling to the nucleus (piggy-back mechanism).

Recently, we reported the association of ZO-2 to transcription factors Jun, Fos and C/EBP. We also showed that the over-expression of ZO-2 inhibited the activity of a reporter gene regulated by AP-1 promoters [18]. Here, we illustrate how the amino ZO-2 segment is in the reporter gene assay, even more effective than the full-length ZO-2, probably due to its higher expression at the nucleus. The diminished response obtained with the mutant amino ZO-2 segments, in comparison with the complete amino construct, reveals that to modulate gene expression, not only the arrival to the nucleus is important, but also the presence of particular domains in the construct. Thus, we suspect that the presence of the second PDZ augments the inhibitory function of ZO-2 upon AP-1-controlled promoters. We previously reported that the 3PSG segment of ZO-2 exerts no effect upon CAT expression, although in a pull- down assay it interacts, albeit weakly, with Fos transcription factor [18]. Now this result can be explained by the observation that the 3PSG segment is not expressed in the nucleus. In contrast, we had reported that the AP domain inhibits CAT activity. This result can be reinforced by the observation, that this protein, although containing no NLS, is capable of effectively reaching the nucleus, probably by associating to transcription factors Jun, Fos and C/EBP as previously reported by us [18].

On analyzing the sequence of ZO-2 we previously detected the presence of two putative NES, one within the second PDZ domain and the other at the GK region of the molecule [8]. Here, we have explored whether such signals are functional by exploring their capability of exporting chemically conjugated ovalbumin. Our results indicate that while the second NES of ZO-2 works as a functional export signal, the first one does not. This result conflicts with recent work on MDCK cells showing that a transfected amino ZO-2 construct containing NES-1 but not NES-2 exports the nucleus [24]. However, since in that study ZO-2 is able to leave the nucleus even when the construction lacks both export signals, one might consider the possibility that ZO-2 is also able to leave the nucleus by associating to other molecules bearing NES. Alternatively, ZO-2 could be leaving the nucleus through its possible association with nuclear mRNA. As we previously observed [8], the first link of the molecule is particularly enriched in R, RG and SRG residues which in many proteins mediate interactions with nuclear mRNA and ribosomal RNA at the nucleolus [43,44].

Acknowledgments

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The Polarized Expression of Na^+,K^+-ATPase in Epithelia Depends on the Association between β-Subunits Located in Neighboring Cells

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The polarized distribution of Na^+,K^+-ATPase plays a paramount physiological role, because either directly or through coupling with co- and countertransporters, it is responsible for the net movement of, for example, glucose, amino acids, Ca^{2+}, K^+, Cl^-, and CO_3H^- across the whole epithelium. We report here that the β-subunit is a key factor in the polarized distribution of this enzyme. 1) Madin-Darby canine kidney (MDCK) cells (epithelial from dog kidney) express the Na^+,K^+-ATPase over the lateral side, but not on the basal and apical domains, as if the contact with a neighboring cell were crucial for the specific membrane location of this enzyme. 2) MDCK cells cocultured with other epithelial types (derived from human, cat, dog, pig, monkey, rabbit, mouse, hamster, and rat) express the enzyme in all (100%) homotypic MDCK/MDCK borders but rarely in heterotypic ones. 3) Although MDCK cells never express Na^+,K^+-ATPase at contacts with Chinese hamster ovary (CHO) cells, they do when CHO cells are transfected with β_1-subunit from the dog kidney (CHO-β). 4) This may be attributed to the adhesive property of the β_1-subunit, because an aggregation assay using CHO (mock-transfected) and CHO-β cells shows that the expression of dog β_1-subunit in the plasma membrane does increase adhesiveness. 5) This adhesiveness does not involve adherens or tight junctions. 6) Transfection of β_1-subunit forces CHO-β cells to coexpress endogenous α-subunit. Together, our results indicate that MDCK cells express Na^+,K^+-ATPase at a given border provided the contacting cell expresses the dog β_1-subunit. The cell–cell interaction thus established would suffice to account for the polarized expression and positioning of Na^+,K^+-ATPase in epithelial cells.

INTRODUCTION

The membrane enzyme Na^+,K^+-ATPase of epithelial cells serves two different but integrated roles. The first is the translocation of ions across the plasma membrane as in other cell types (Skou, 1957; Skou, 1998). The second stems from its expression in a particular domain of the plasma membrane (polarization), in such a way that it propels the translocation of Na^+ across the whole epithelium as proposed by Koefoed-Johnsen and Ussing (1958). In turn, a combination between the polarized distribution of Na^+,K^+-ATPase and the polarized expression of co-, countertransporters, and ion channels drives the net transport of, for example, glucose, amino acids, Ca^{2+}, K^+, Cl^-, and CO_3H^- across epithelia (Schultz and Curran, 1969; Cereijido and Rotunno, 1971; Rabito and Karish, 1983). In keeping with these roles, Na^+,K^+-ATPase is found to reside on the basolateral surface in most epithelial cells (Cereijido *et al.*, 1980; Ernst and Mills, 1980; Fambrough and Bayne, 1983; Kashgarian *et al.*, 1985). In a few other epithelial cells, such as those of the choroid plexus (Wright, 1972), retinal pigment epithelium (Steinberg and Miller, 1979; Gundersen *et al.*, 1991), and cockroach salivary gland (Just and Walz, 1994), this enzyme is expressed on the opposite side of the cells but always in a strictly polarized manner.

Na^+,K^+-ATPase is a heteromultimer comprised of three subunits. The 110-kDa α-subunit bears the Mg^{2+}, ATP, Na^+, K^+, and ouabain binding sites and is therefore considered to be the catalytic subunit of the enzyme. The β-subunit is a glycoprotein of 40–60 kDa that seems to be involved in the structural and functional maturation of the holoenzyme (Geering *et al.*, 1989; Ackermann and Geering, 1990) and subsequent transport of the α-subunit to the plasma membrane (Noguchi *et al.*, 1987; Fambrough, 1988; Takeyasu and Kawakami, 1989). Ion transport requires the participation of both α- and β-subunits (Noguchi *et al.*, 1987; Horowitz *et al.*, 1990). These units assemble during or soon after biosynthesis (Fambrough and Bayne, 1983), a requirement to exit from the endoplasmic reticulum (Jaunin *et al.*, 1992). Finally, there is a small γ-subunit (Forbush *et al.*, 1978) that belongs to the FXYD family that modulates Na^+,K^+-ATPase activity (Sweadner *et al.*, 2000); it is not referred to in the present work.

Although for macroscopic processes such as net transepithelial fluxes and electrical potentials it is irrelevant whether the pump is located at the basal or at the lateral side of the cells, the results of the present work suggest that the expression on the lateral plasma membrane facing the intercellular spaces may be decisive for the polarized distribution

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Table 1. Percentage of expression of Na^+,K^+-ATPase in MDCK cells contacts

Cell line	Animal species	Organ	Cell morphology	ATPase
MDCK	Dog	Kidney	Epithelial	100
CRFK	Cat	Kidney	Epithelial	45
CF2TH	Dog	Thymus	NA	37
PTK_2	Marsupial rat	Kidney	Epithelial	10
$LLCPK_1$	Pig	Kidney	Epithelial	6
Ma104	Monkey	Kidney	Epithelial	0
LLCMK2	Monkey	Kidney	Epithelial	0
NRK E52	Rat	Kidney	Epithelial	0
VERO	Monkey	Kidney	Epithelial	0
293	Human fetal	Kidney	Epithelial	0
CHO	Chinese hamster	Ovary	Epithelial	0
COS-7	Monkey	Kidney	Fibroblast	0
3T3	Mouse	Embryo	Fibroblast	0

Cells listed in the first column were labeled with CMTMR, mixed in 50:50 proportions with MDCK cells, plated at confluence and incubated overnight. Monolayers were then fixed, treated with a first antibody against the dog β_1-subunit, and a second, fluoresceinated one. These were then observed by confocal microscopy as described in Figure 1. One or two hundred borders between MDCK/other cell type were analyzed and scored positive if they exhibited green fluorescence staining. Last column on the right shows the proportion of heterotypic borders exhibiting β_1-subunit (except for the first line, where MDCK/MDCK have no heterotypic contacts). This subunit of Na^+,K^+-ATPase was present in 100% of the homotypic MDCK/MDCK contacts and in a lower proportion in heterotypic ones. NA, not available.

of the pump. We study this polarized expression in MDCK cells. We describe the lateral expression of Na^+,K^+-ATPase in pure MDCK monolayers and in coculture with other cell types, in particular when this other cell type has been transfected with the dog β_1-subunit. We observe that the expression of this subunit in Chinese hamster ovary fibroblasts (CHO) cells up-regulates the coexpression of endogenous α-subunit at the cell surface, confers cell adhesiveness, and induces the expression of the pump in heterologous contacts when cocultured with MDCK. Together, these results indicate that the β-subunit plays a major role in the polarized expression of Na^+,K^+-ATPase.

The present results were partially presented in overall reviews (Cereijido *et al.*, 2000, 2001, 2003, 2004; Shoshani and Contreras, 2001).

MATERIALS AND METHODS

Cell Culture

Starter cell cultures were obtained from the American Type Culture Collection (MDCK, CCL-34; LLC-PK1 CRL 1392; LLC-MK2, CCL-7; NRK-52E, CRL-1571; PtK2, CCL-56; VERO, CCL-81; COS-7, CRL-1651;CRFK, CCL-94; CF2TH, CRL-1430; 3T3, CL-173; and CHO, CCL-61; 293, CRL-1573). Ma104 cells (epithelial line derived from rhesus monkey kidney) were a generous gift from Dr. E. Rodríguez-Boulán (Cornell University, Ithaca, NY). MDCK and Ma104 cells were cloned, and all experiments reported here were performed in cells of clone 7 and 1, respectively. All cells were grown at 36.5°C in disposable plastic bottles (3250; Costar, Cambridge, MA) in a 5% CO_2 atmosphere (Forma Scientific CO_2 incubator, Steri-Cult 200). We used DMEM (430-1600; Invitrogen, Carlsbad, CA), with 100 U/ml penicillin, 100 μg/ml streptomycin (600-5145; Invitrogen), 0.8 U/ml insulin (Eli Lilly, Indianapolis, IN), and 10% fetal calf serum (FCS) (200-6170; Invitrogen), except for CHO cells that were cultured in a mixture of F12/DMEM. Cells were harvested with trypsin-EDTA and plated on dishes with or without glass coverslips.

Plasmid Construct and Transfection

The full-length cDNA of dog kidney Na^+,K^+-ATPase β_1-subunit (cloned into $pKS^{+\beta\Delta}11$; a generous gift from Dr. R. Farley, University of Southern California School of Medicine, Los Angeles, CA) was subcloned into the expression vector pIRESneo (previously known as pCIN4 from BD Biosciences Clontech, Palo Alto, CA). Briefly, $pKS^{+\beta\Delta}11$ was restricted with *Spe*I and *Sac*I, and the insert corresponding to the full-length β_1 was then blunt end ligated to the pCIN4 vector that was previously restricted with *Eco*RV. Two positive clones of transformed bacteria were subjected to plasmid purification with QIAGEN plasmid midi kit (catalog no. 12143; QIAGEN, Valencia, CA). The transfection was as follows: CHO cells were plated at 90% density on glass coverslips placed in a culture dish of 3 cm in diameter, in a serum-free medium. Cells were then incubated for 5 h with a mixture of LipofectAMINE Plus (10964-013; Invitrogen) and 3 μg of the purified plasmid (pCIN4 [mock] or pCIN4-β), as indicated by the manufacturer. Thereafter, the transfection medium was removed, and the cells were incubated for additional 24 h with fresh serum-containing medium to allow recovery. Subsequently, the coverslips were removed and processed for immunofluorescence, to confirm the transient expression. Then, 0.8 mg/ml G418 was added to select stable clones expressing the dog Na^+,K^+-ATPase β_1-subunit. Except for biotinylation and Takeichi's aggregation assays, in which various clones have been compared, all the experiments were done with clone 10c that was chosen because it expresses the dog Na^+,K^+-ATPase β_1-subunit mainly in the plasma membrane. Stable clones are maintained with 0.2 mg/ml G418 in F12/DMEM mixture.

Mixed Monolayers

Cell mixture was performed as described previously (Contreras *et al.*, 1995; Cereijido *et al.*, 2002). Briefly, usually the cell type different from MDCK (CHO, CHO-β, Ma104, and normal rat kidney [NRK]) was prelabeled with 5-(and 6-)[[(4-chloromethyl)benzoyl]amino]-tetramethylrhodamine (C-2927, CellTracker Orange CMTMR; Molecular Probes, Eugene, OR). This was achieved by incubating the cells for 1 h at 36.5°C, with CMTMR in dimethyl sulfoxide added to the medium to a final concentration of 6.3 μM. Cells were then washed three times with phosphate-buffered saline (PBS) solution, reincubated for 1 h in DMEM supplemented with 10% FCS. Then, the cells are trypsinized and the suspension is mixed with MDCK cells suspension, in equal parts, to be cocultured on glass coverslips and processed the day after for immunofluorescence assay.

Immunofluorescence Microscopy

For immunofluorescence microscopy, cells grown on coverslips were washed with PBS and then fixed and permeabilized with ice-cold methanol for 5 min. After washing with PBS, the cells were soaked in blocking solution (PBS containing 5% fetal bovine serum) for 1 h. The cells were then incubated with the first antibodies for 30 min at 37°C, washed 10 times quickly with PBS, and thereafter incubated with the secondary antibodies for 30 min at room temperature. All antibodies were diluted in blocking solution, and the following secondary antibodies were used: Alexa 488-conjugated goat anti-mouse IgG, (Molecular Probes) and CY5-goat anti-rabbit IgG (Zymed Laboratories, South San Francisco, CA). After washing, as indicated previously, specimens were mounted on glass slides with FluoroGuard antifade reagent (170–3140; Bio-Rad, Hercules, CA) and observed with a confocal microscope (Bio-Rad MRC-600 or Leica TCS SP2) (Leica, Hiena, Germany). The β_1-subunit of the Na^+,K^+-ATPase was identified with a mouse monoclonal antibody donated by Dr. M. Caplan (Yale University, New Haven, CT), and the α-subunit with a polyclonal antibody (F592-594) donated by Dr. D. Fambrough (The Johns Hopkins University, Baltimore, MD). Acquisition and analysis were performed with the respective software (COMOS, Bio-Rad: LCS, Leica) and with Image J from National Institutes of Health (Bethesda, MD).

Immunoblot

All the extraction steps are performed at 4°C. Monolayers grown on 3-cm dish were washed three times with PBS, and then 200 μl of RIPA buffer containing 10 mM PIPES, pH 7.4, 150 mM NaCl, 2 mM EDTA plus 1% Triton X-100, 0.5% DocNa, 10% glycerol, and protease inhibitors (Complete, Mini; Roche Diagnostics, Indianapolis, IN) was added. The cells were scrapped with rubber policeman, and the cell lysate collected into a 1.5-ml microcentrifuge tube. This was repeated with another 200 μl of RIPA and then incubated for 30 min under continuous and vigorous shaking. The extract was sonicated for 30 s and centrifuged 20 min at 14,000 rpm in a microcentrifuge. The supernatant was recovered, and its protein content was measured with the BCA protein assay reagent (catalog no. 23225; Pierce Chemical, Rockfield, IL) and subsequently boiled in Laemmli sample buffer. The SDS-PAGE–resolved proteins were electrotransferred to polyvinylidene difluoride (PVDF) membrane (RPN 303F, Hybond-P; Amersham Biosciences, Piscataway, NJ), which were blocked with 5% dry defatted milk and 3% bovine serum albumin (BSA) in PBS. The proteins of interest were detected with the specific polyclonal or monoclonal antibodies indicated above, followed by species-appropriate peroxidase-conjugated antibodies (62-6120 and 62-6520, Zymed Laboratories; and A-9037, Sigma-Aldrich, St. Louis, MO) and a chemiluminescent detection system (RPN2132, ECL PLUS; Amersham Biosciences).

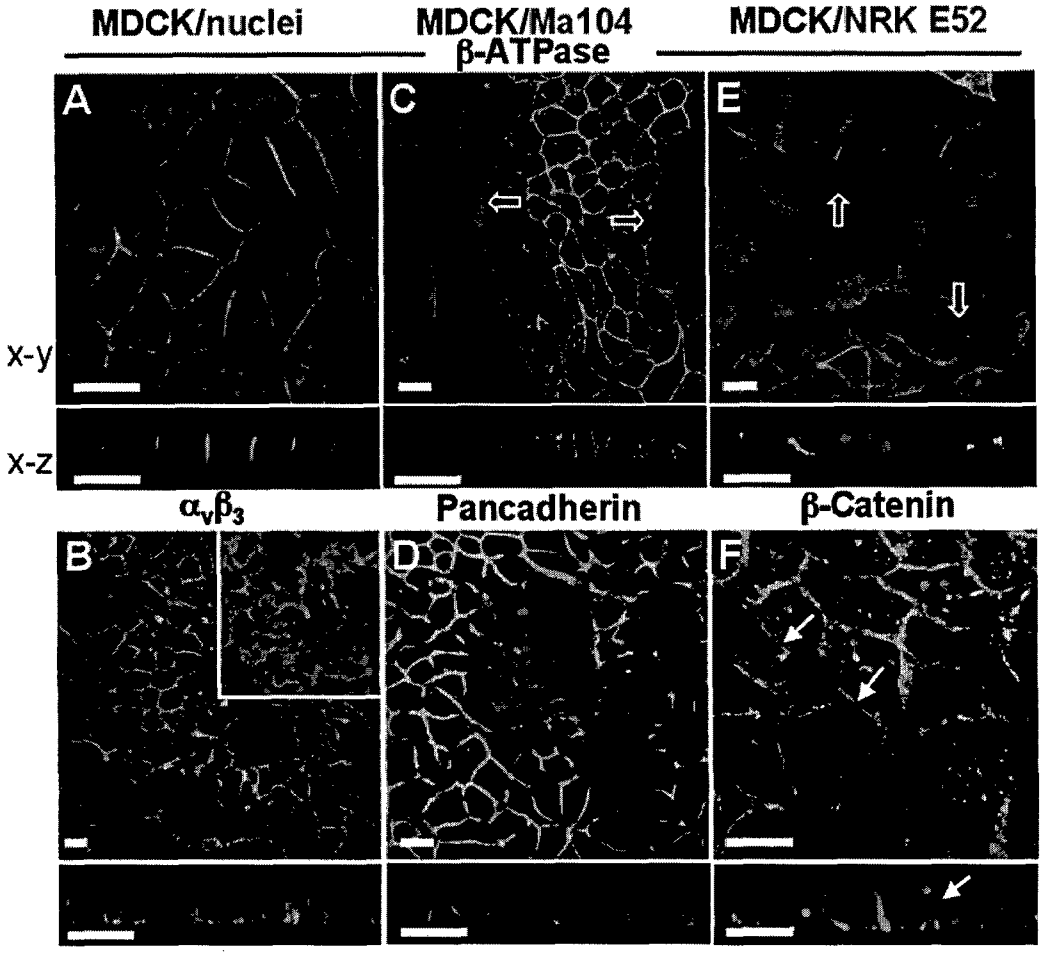

Figure 1. MDCK cells express β_1-subunits at homotypic but not at heterotypic contacts. Localization of the β_1-subunit (A, C, and E, green) of Na^+,K^+-ATPase and cell adhesion proteins (B, D, and F, green) was studied by immunofluorescence assay in MDCK cells derived from dog kidney. (A) Monolayer of pure MDCK cells with nuclei stained with propidium iodide (red) shows that the β_1-subunit is only expressed at the plasma membranes in the lateral domain where cells contact each other. (B) Integrin $\alpha_V\beta_3$ (green) shows the staining pattern of a membrane protein that is expressed at the lateral as well as the basal membrane domain (inset). (C) MDCK cells (unstained) cocultured with Ma104 ones (derived from monkey kidney) that were labeled beforehand with CMTMR (red). β_1-Subunit in mixed monolayer is only expressed in homotypic borders (MDCK/MDCK) but not in heterotypic ones (MDCK/Ma104, empty arrows). (D) Pancadherin antibody (green) staining a conserved sequence of cadherins shows that these molecular species are present at all cell borders in mixed monolayers, regardless of whether this contact is homo- or heterotypic. (E) Monolayer of mixed MDCK/NRK-E52 cells (epithelial line derived from normal rat kidney, red) showing that β-subunit (green) concentrates in homotypic MDCK/MDCK contacts, whereas β-catenin, an adherent junction marker, is clearly observed in all cell-cell contacts (F, filled arrows). Bars, 20 μm.

Biotinylation of Cell Surface Proteins

Confluent monolayers of stable clones of CHO cells expressing the dog β_1-subunit or the empty vector were rinsed twice with 5 ml of ice-cold PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (CM-phosphate-buffered saline), followed by the addition of 3 ml of the same ice-cold solution containing 0.5 mg/ml freshly added SULFO-NHS-SS-biotin (Pierce Chemical) for 45 min. Fresh buffer and biotin were added and incubated another 45 min. For quenching, the plates were washed three times with CM-phosphate-buffered saline containing 100 mM glycine. The cells were scraped in RIPA buffer for 10 min at 4°C, and the homogenates were centrifuged at 14,000 rpm, for 10 min. Protein of each lysate was used for precipitation (16 h at 4°C) with 30 μl of Neutravidin beads (Pierce Chemical). The beads were washed four times with RIPA buffer, and the final pellets were resuspended in 100 μl of 4× Laemmli buffer and incubated 1 h at 37°C. The beads were pelleted, and the solubilized proteins were separated by SDS-PAGE, transferred to PVDF membranes and probed with antibodies directed against α- and β-subunits of the Na^+,K^+-ATPase as indicated in Immunoblot.

Aggregation Assays

CHO cells stably expressing dog β_1-subunit or the empty vector and MDCK cells (as controls) were tested for their ability to aggregate with two different techniques: hanging drop suspension cultures and classical cell aggregation assay.

Hanging Drop Suspension Cultures (Thoreson and Reynolds, 2002). Cells were trypsinized in the presence of EDTA, washed twice in PBS, and resuspended in F12/DMEM without serum. Cells (1.5×10^4) in 30 μl of media were suspended as hanging drops from the lid of a 24-well culture dish and allowed to aggregate overnight in a humid 5% CO_2 incubator at 37°C. Corresponding wells were filled with PBS to prevent drying of the drops. Aggregation was evaluated 14–18 h after plating. To assay for tightness of cell-cell adhesion, cells were subjected to shear force by passing them 10 times through a standard 200-μl micropipette tip. Cells were observed through a light microscope with 5× phase contrast objective (DMIRE2; Leica). For quantification, after the pipetting stress, pictures (DC-300F; Leica) of individual fields of cells were scored for small (7–20 cells) or large (>20 cells) aggregates. The data presented here are from three experiments in which 12 pictures were analyzed for each cell type. Results are expressed as mean ± SE.

Classical Cell Aggregation Assay (Takeichi, 1977). Confluent monolayers were treated with 0.2% (wt/vol) trypsin and 1 mM EDTA at 37°C for 5 min and dispersed by moderate pipetting. Cells were resuspended in P buffer (145 mM NaCl, 10 mM HEPES, pH 7.4, 1.0 mM Na-pyruvate, 10 mM glucose, 3.0 mM $CaCl_2$) complemented with Complete Mini (Roche Diagnostics) at 10^6 cells/ml, except for the Ca^{2+}-dependent experiments in which DMEM with (1.8 mM) or without (5 μM) Ca^{2+} was used. Cell suspension was placed in 1.5-ml microfuge tubes precoated with BSA and rotated on a gyratory shaker

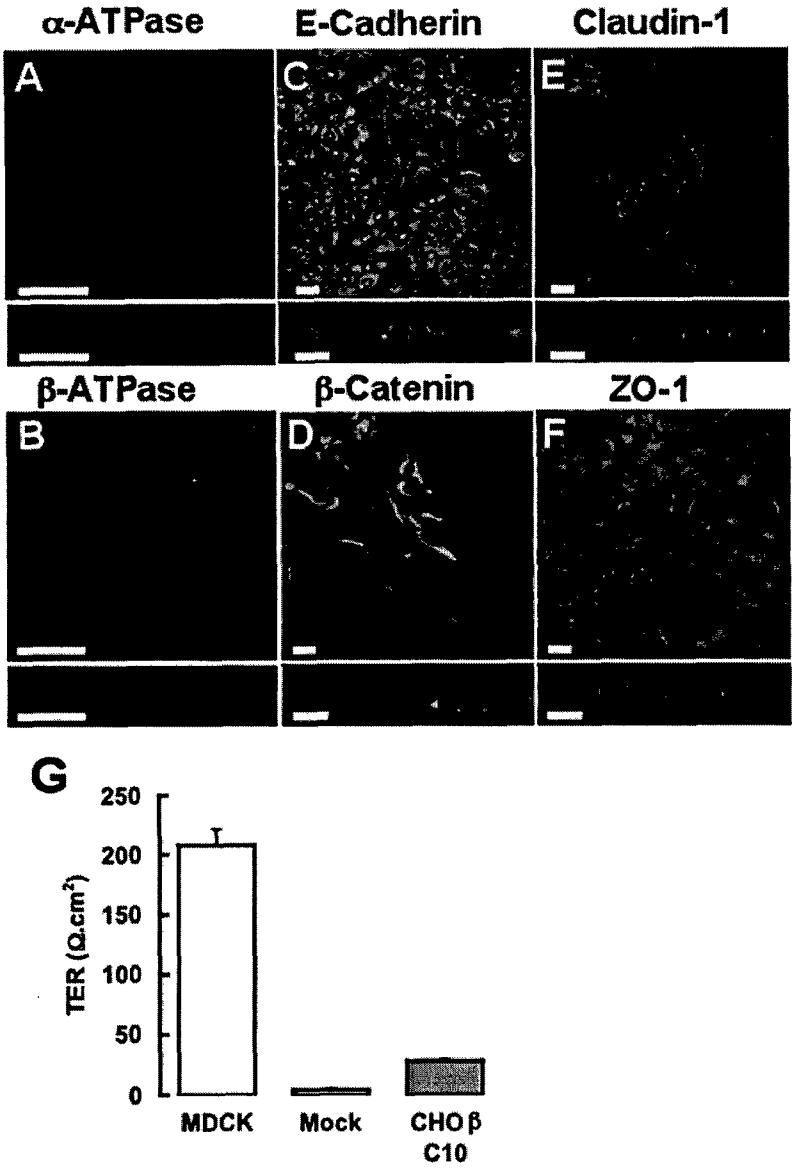

Figure 2. Absence of physiological and molecular evidence for tight and adherent junctions in wild CHO cells cultured in pure and mixed monolayers. Immunoassays of α- and β-subunits of Na^+,K^+-ATPase in pure monolayers of CHO cells (nuclei stained in red with propidium iodide) show no evidence of this enzyme (A and B). (C–F) Mixed monolayers of MDCK and CHO cells (red). E-cadherin (C) and β-catenin (D), which are specific markers of epithelial adherent junctions, as well as claudin-1 (E) and ZO-1 (F) corresponding to tight junctions, are only present at MDCK borders (green). (G) TER of monolayers of MDCK cells, mock-transfected CHO cells, and CHO-β. Bars, 20 μm.

at 37°C for 3 h. Aggregation was stopped by adding 2% (vol/vol) glutaraldehyde. The extent of aggregation was assessed by fluorescence-activated cell sorting (FACS) analysis of 50,000 events (FACS Vantage; BD Biosciences, San Jose, CA).

Transepithelial Electrical Resistance (TER)

The degree of sealing of the tight junctions was assessed by measuring the transepithelial electrical resistance (TER) (Cereijido *et al.*, 1978, 2002). After incubation under a given condition, the filter with the monolayer was mounted as a flat sheet between two Lucite chambers with an exposed area of 0.69 cm^2. Current was delivered via Ag/AgCl electrodes placed at 2.0 cm from the monolayer; the voltage deflection elicited was measured with a second set of electrodes placed at 1.0 mm from the membrane. Values of TER reported were obtained by subtracting the contribution of the filter and the bathing solution. A given monolayer was used only for a single determination and discarded to avoid leaks due to edge damage.

RESULTS

The Polarized Distribution of Na^+,K^+-ATPase

Studies with radioactive tracers, short-circuit currents, sidedness of the effect of inhibitors, and [^{3}H]ouabain labeling had long anticipated that the Na^+,K^+-ATPase would be found on the basolateral side (Cereijido *et al.*, 1980, 1981, 2000, 2001; Louvard, 1980; Rabito and Tchao, 1980; Contreras *et al.*, 1989). Yet, the study of the distribution of this enzyme in at least 100 contacts (in each case) of confluent monolayers of epithelial cells by using immunofluorescence approach, provides some revealing details on this localization (Table 1 and Figure 1). Foremost, the enzyme is localized at the ***lateral*** border of the cell (Figure 1A) and not at the ***baso***lateral one. Figure 1B showing $\alpha_v\beta_3$-integrin staining at lateral as well as basal domains demonstrates that the confocal method used is able to detect a protein placed at this border and that the absence of β_1-subunit at the basal membrane may not be attributed to a technical difficulty. The lateral expression of this subunit seems to depend on cell-cell contacts, as well as the nature of the neighboring cell. Thus, when this neighbor is another MDCK cell, expression of Na^+,K^+-ATPase is observed in 100% of the contacting borders. This percentage markedly decreases in contacts with other cell lines (Table 1). Actually, many borders that we have scored as positive in heterotypic contacts were in

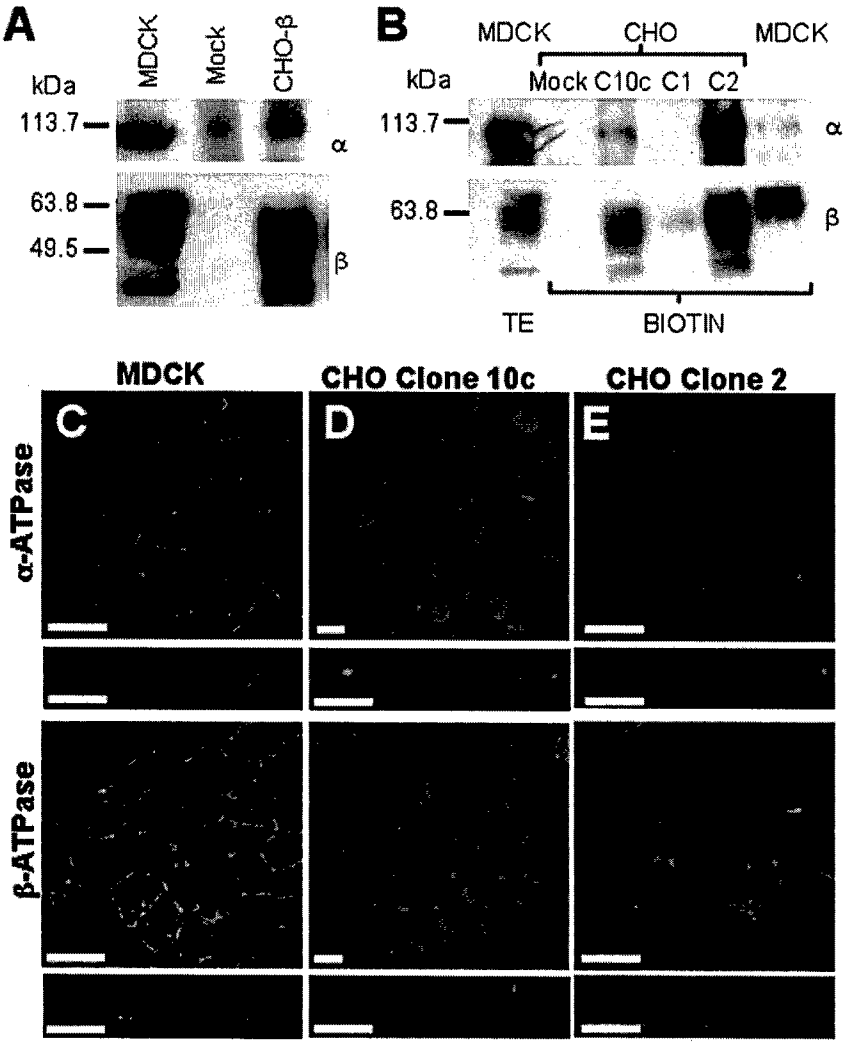

Figure 3. Expression of α- and β_1-subunits in epithelial (MDCK) and fibroblastic CHO cells. (A) Western blots using an antibody against α-subunit (α) and dog β_1-subunit (β). As expected, MDCK cells show a heavy mark due to glycosylated β_1-subunits. Mock-transfected CHO cells do not show a β_1-subunit band, whereas transfected ones (CHO-β) exhibit two bands corresponding to β_1-subunits. Blotting with anti-α antibody shows heavy staining in MDCK cells. The antibody cross-reacts with the α-subunit of CHO cells. As expected, CHO-β cells exhibit a much heavier staining. (B) Western blots of biotinylated α- and β1-subunits expressed on the cell surface. MDCK lane shows the usual migration pattern of both subunits of total extracts (TE) and cell surface extracts (BIOTIN). Mock-transfected CHO cells show no signal, whereas three different clones of CHO cells transfected with the dog β_1-subunit (C10c, C1, and C2) express different levels of the exogenous protein at cell surface. Immunofluorescence confocal images of MDCK (C), 10c clone (D), and clone 2 of CHO-β (E) stained against α- and β-subunits of Na+,K+-ATPase. Each image is accompanied by the transverse section. Nuclei were stained with propidium iodide (red). MDCK cells show the typical chicken fence pattern of both subunits. Transverse sections show the exclusive lateral distribution of the Na+,K+-ATPase. CHO-β clones express the transfected dog β1-subunit as well as the endogenous α-subunit in the plasma membrane. Transverse optical sections below indicate that the Na^+,K^+-ATPase in CHO-β is not polarized. Bars, 20 μm.

fact due to faint labeling presumably caused by immature contacts or rearrangement of cells in the monolayer. Not even the fact that the neighboring cell derives from the same animal species (e.g., dog thymus CF2TH cells) or from the same organ (e.g., kidney) of other animal species (e.g., PTK_2, $LLCPK_1$, and NRK-E52) ensures that MDCK cells would express Na^+,K^+-ATPase in the heterotypic border. A given MDCK cell that expresses its β-subunit in a homotypic contact does not express it on the other side, when this side contacts a rat kidney cell (Figure 1E, NRK-E52). The same observation is repeated with Ma104 (Contreras *et al.*, 1995; Figure 1C), LLCMK2, human embryonic kidney 293, VERO, and CHO cells. This absence of expression of Na^+,K^+-ATPase in heterotypic contacts between epithelial cells may not be attributed to lack of intimate cell adhesion. Thus, Figure 1, D and F, shows in two different cocultures (MDCK/Ma104 and MDCK/NRK-E52) the expression of cadherin (anti-pancadherin antibody) and β-catenin in both homotypic and heterotypic borders. This is in agreement with our previous results demonstrating that monolayers of mixed epithelial cell types have the TER that could be expected from the TER of each cell type in pure monolayers, and their proportion in the mixture (Gonzalez-Mariscal *et al.*, 1989; Contreras *et al.*, 2002). Furthermore, mixed and pure monolayers had the same structure and arrangement of TJ strands in freeze fracture replicas (Gonzalez-Mariscal *et al.*, 1989). Contreras *et al.* (2002) have shown that these heterotypic contacts also express molecules such as E-cadherin, ZO-1, and occludin. On the contrary, we were unable to detect Na^+,K^+-ATPase in contacts with cells lines of fibroblastic morphology such as 3T3 and COS-7.

Overexpression of β-Subunit in CHO Cells

To study the role of the β-subunit in the polarized expression of Na^+,K^+-ATPase, we selected CHO cells, because MDCK cells do not express a detectable amount of Na^+,K^+-ATPase in its heterotypic borders with CHO (Table 1). As shown in Figure 2, endogenous Na^+,K^+-ATPase in CHO cells cannot be detected with anti-dog antibodies, an observation that is repeated with adhesion-associated proteins such as E-cadherin (Figure 2C), β-catenin (Figure 2D), claudin-1 (Figure 2E), or ZO-1 (Figure 2F), which are readily observed in MDCK cells. Furthermore, when cultured as layers on permeable supports, no considerable electrical resistance is observed (Figure 2G). Accordingly, we transfected CHO cells with the cDNA of dog β_1-subunit and obtained stable clones (CHO-β; see *Materials and Methods*). Figure 3A shows that protein extracts of CHO-β cells assayed with an antibody against the dog β_1-subunit exhibit a major glycosylated band (~50 kDa) similar to the one de-

tected in MDCK cells. This antibody does not recognize any β-subunit in the mock-transfected CHO fibroblasts. On the other hand, an antibody against the α-subunit of Na^+,K^+-ATPase that recognizes a C-terminal conserved motif, shows the typical ~110-kDa band in the Western blot corresponding to α-subunit. Interestingly, the expression of endogenous α-subunit in CHO fibroblast is very low (Figure 3A, mock). Nevertheless, in CHO-β cells the expression of α-subunit is more pronounced, resembling that of MDCK cells. This could either reflect an increase in a gene expression or increased stability of synthesized α-subunit that is now able to reach the plasma membrane. To assess whether the expression of α-subunit induced by the overexpression of β_1-subunit reaches the cell surface, we used a biotinylation assay (Figure 3B). Mock-transfected CHO cells do not express α- nor β_1-subunit in their plasma membrane, whereas the different clones of CHO-β (C10, C1, and C2) express both of them at the surface. These findings are in accordance with the recent study from the Rajasekaran laboratory (Rajasekaran *et al.*, 2004) indicating that in mammalian cells, the Na^+,K^+-ATPase β_1-subunit is involved in facilitating the translation of the α_1-subunit mRNA in the endoplasmic reticulum. Observations with immunofluorescence microscopy of MDCK, CHO, and CHO-β cells are in keeping with the biotinylation data. Thus, MDCK cells show a plasma membrane codistribution of both subunits (Figure 3C). Transverse optical sections confirm that the expression of Na^+,K^+-ATPase occurs mostly at the lateral borders. Mock-transfected CHO cells show no membrane staining (Figure 2A), whereas CHO-β cells (clone 10c and clone 2, Figure 3, D and E, respectively) show that both α- and β_1-subunits are localized at the plasma membrane, even though a green staining spread throughout the cytoplasm is observed. This may be due to overexpressed protein that is trapped in intracellular compartments. Nonetheless, our observations suggest that the transfection of β_1-subunit in CHO cells up-regulates the expression of the endogenous α ones.

Polarization of Na^+,K^+-ATPase in CHO Fibroblasts

Previous studies of various groups suggested that nonpolarized cells are capable of polarized plasma membrane delivery, but they lack the spatial segregation of distinct membrane targets (Musch *et al.*, 1996; Yoshimori *et al.*, 1996). Therefore, CHO-β fibroblasts were not expected to deliver the pump to the plasma membrane in a polarized manner unless appropriate extrinsic signals and asymmetric plasma membrane cues are established, leading to a differentiated apical/basolateral membrane domains formation (Shoshani and Contreras, 2001; Cereijido *et al.*, 2003). Indeed, the transversal optical sections of transfected monolayers in Figure 3, D and E, indicate that the pump distribution in CHO-β cells is not polarized. Moreover, in transfected CHO-β cells (Figure 4, green) cocultured with mock-transfected CHO cells that were labeled beforehand with CMTMR (red), the plasma membrane expression of the β_1-subunit is observed in both contacting and noncontacting cell borders, and, again, it is not restricted to a particular pole of the cell.

Transfection of the β_1-Subunit Confers Cell Adhesiveness

Fibroblastic CHO-β cells tend to form continuous layers, as if they establish an intimately registered vicinity (Figure 3D). Because these cells were not cotransfected with E-cadherin and do not exhibit a significant electrical resistance across themselves ($28.3 \pm 1.5\ \Omega \cdot cm^2$; Figure 2G), it could imply that β-subunit is acting as an adhesion molecule, as observed by Gloor *et al.* (1990) in glial cells. Therefore, to test the adhesiveness conferred by the β-subunit to CHO cells, we used the two aggregation assays described in *Materials and Methods*. In the first method (Thoreson and Reynolds, 2002), CHO-β cells (Figure 5A) associate in small (7–20 cells) or large (>20 cells) aggregates in a higher proportion than mock-transfected cells (Figure 5B) (136 and 380% increase for small and large aggregates, respectively; $p < 0.001$) (Figure 5C). MDCK cells under the same experimental conditions clump spontaneously (Figure 5D), except when the medium was devoid of Ca^{2+} (Figure 5E). To ensure that the adhesiveness of CHO-β cells (clone 10c) does not depend on the chosen clone, we performed a comparative aggregation assay (Takeichi, 1977) followed by FACS analysis to four separate β_1-subunit expressing CHO-β clones and MDCK cells. Our data reveal that the distribution of aggregates of β_1-transfected clones resembles that of MDCK cells. All clones exhibit at least a 100% increase in adhesiveness with respect to mock-transfected CHO cells, as reflected by the relative density in the top right quadrant of the plot (Figure 5F). Furthermore, the adhesiveness in the absence (5 μM) or presence (1.8 mM) of Ca^{2+}, of mock-transfected (mock) and β_1-transfected CHO cells (C10c) also was analyzed by FACS (Figure 5G). The relative density in the top right quadrant of the plot, of mock-transfected cells (0.98 and 0.68%, respectively) and C10c cells (11.35 and 10.8%, respectively) suggests that adhesiveness does not depend on extracellular Ca^{2+}. Together, these assays show that the β-subunit does confer adhesiveness to the cells.

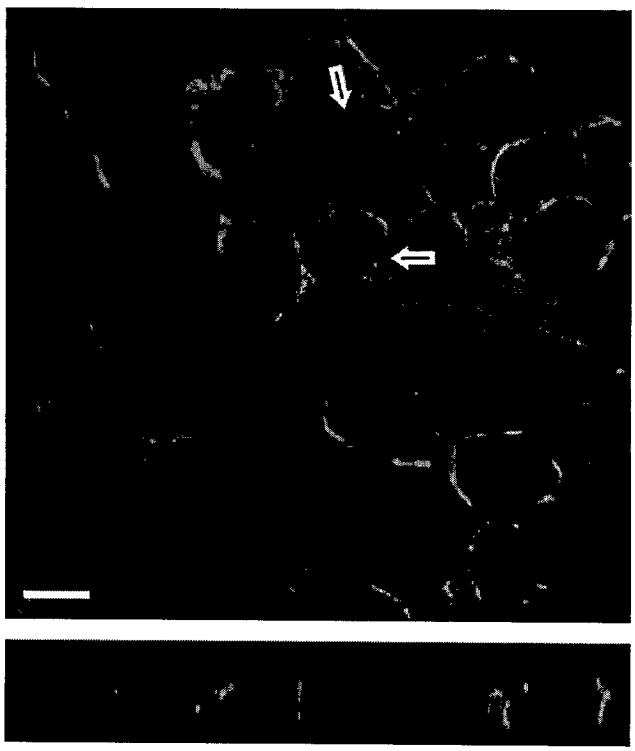

Figure 4. CHO fibroblasts (red) cocultured with CHO-β stably expressing the β_1-subunit (green) of dog Na^+,K^+-ATPase. Fibroblasts, either wild or transfected do not segregate from each other. CHO-β cells express the dog β_1-subunit at the membrane in hetero- (empty arrow) and homotypic contacts, as well as free borders. Optical transverse sections (below) show the expression of β_1-subunit all around the cell, confirming that fibroblasts do not express this protein polarizedly. Bars, 10 μm.

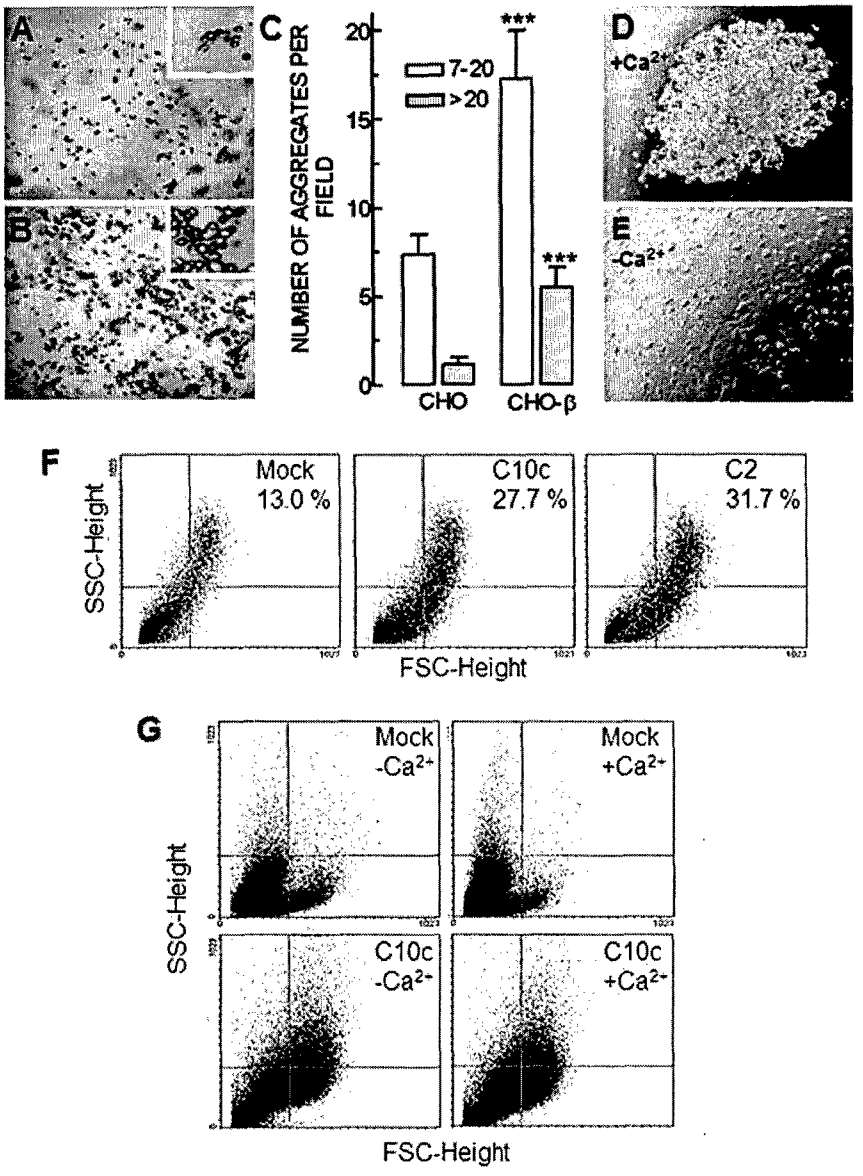

Figure 5. Cell aggregation assay. The adhesiveness of CHO cells transfected with the β-subunit was assayed following the two different aggregation assays described in *Materials and Methods*. (A) CHO cells transfected with the empty vector (mock) are mostly isolated or in small aggregates (inset). (B) CHO-β cells tend to aggregate in larger clumps (inset). (C) Clumps were scored into two groups: those with 7–20 cells (white bars) and those larger than 20 cells (gray bars). Groups of less than seven cells are not represented. The first two columns correspond to mock-transfected CHO cells that tend to group in small clumps of 7–20 cells. Transfection of β-subunit (last two columns) produces a 136% increase in the number of small clumps ($p < 0.001$) and a 380% in the number of large clumps ($p < 0.001$). Each column represents an average of 36 individual fields. MDCK cells at the tip of the hanging drop form a large clump in the presence of 1.8 mM of Ca^{2+} (D), whereas in the absence of Ca^{2+} the cells are dispersed (E). (F) Dot plots depicting cell complexity (SSC height) versus cell size (FSC height) of mock- and dog β_1-transfected CHO cells (clones C10c and C2). All β_1-transfected CHO cells exhibited at least a 100% increase in adhesiveness as estimated by the percentage of particles in the top right quadrant of the plot (higher size and complexity). (G) Dot plots of dog β_1- (C10c) and mock-transfected CHO cells in the absence ($-Ca^{2+}$) or presence ($+Ca^{2+}$) of calcium ions in the bathing medium during aggregation assay.

Coculture of MDCK and CHO Cells

If it is true that MDCK cells express the Na^+,K^+-ATPase in homotypic borders because the neighboring cell simultaneously expresses the same type of β-subunit (Figure 6), it is expected that they will express the pump at heterotypic contacts with CHO-β cells. To test this possibility, we mixed the two cell types and observed that heterotypic borders between MDCK and CHO cells that do not express β-subunit from the dog do not show the presence of Na^+,K^+-ATPase (Figure 7A). Yet, MDCK cells cocultured with CHO-β cells do express the pump at both homo- and heterotypic contacts (Figure 7B). To discard the possibility that this expression at heterotypic contacts of MDCK cells were attributed to induction of E-cadherin or other bona fide adhesion-associated proteins in CHO-β cells, we stained the mixed monolayers with antibodies against several adhesion markers. Figure 7, C–F, shows that epithelial adherent proteins (E-cadherin and β-catenin) as well as tight junction proteins (claudin-1 and ZO-1) are not detected in CHO-β cells.

DISCUSSION

Although the asymmetry of epithelia was discovered by Émile Du Bois Raymond in the second half of the nineteenth century, it took the introduction of radioactive tracers, the devise of electrophysiological techniques, and another century to show that epithelia are in fact able to transport net amounts of a given substance all the way across themselves (for a historical sketch, see Cereijido *et al.*, 2003, 2004). Eventually, the Na^+,K^+-ATPase, which had been shown to account for the transport of Na^+ and K^+ across the plasma membrane of individual cells, was found to be also the provider of electrochemical gradients that drive the net movement of sugars, amino acids, and other ion species. Yet to fulfill these transepithelial transports, it is not sufficient that the enzyme is placed at the plasma membrane of epithelial cells, but it also must be present in one of the poles only. This polarization is far from being understood, but several characteristics provide some clues: 1) Na^+,K^+-ATPase is not a basolateral protein as generally assumed, but just

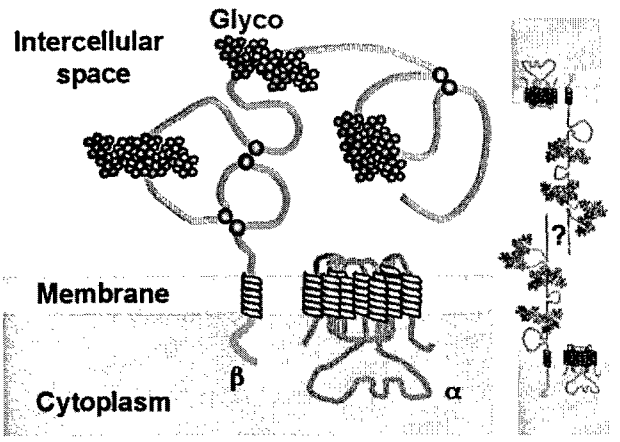

Figure 6. Schematic representation of Na^+,K^+-ATPases located at the lateral border of epithelial cells. The α-subunit with its 10 transmembrane domains is represented. The β-subunit is depicted with a single transmembrane segment and a long extracellular domain containing three S-S links (gray dots) and three glycosylation sites (green). The structure and function of the enzyme requires that both subunits interact closely and strongly; yet for clarity, these are represented as molecules placed far from each other. For the same reason, γ-subunit is omitted. On the right-hand side two β-subunits belonging to neighboring cells are represented as spanning the intercellular space. The present results suggest the possibility that a β-β linkage anchors the Na^+,K^+-ATPase at the lateral borders of epithelial cells. The question mark indicates that we ignore whether the β–β interaction would be a direct one or mediated by an as yet unknown molecule.

lateral (Figure 1). 2) The different isoforms of β-subunits have the typical structure of a cell attachment protein: short cytoplamic tail, a single transmembrane domain, and a long and highly glycosylated extracellular domain (Shull *et al.*, 1986) (Figure 6); 3) in fact, β_2-subunit was found to act as a cell attachment protein (Gloor *et al.*, 1990) that has a high degree of homology with the β_1-isoform expressed by MDCK cells. Accordingly, our first step was to demonstrate that transfected CHO cells do express this subunit (Figure 3A, CHO-β) and that this expression takes place at the plasma membrane (Figure 3B) and confers an adhesiveness that prompts the cells to adapt their borders and adopt an "epithelioid" shape (Figure 3, D and E). Yet, this is not a true epithelial arrangement, because CHO-β layers do not exhibit the typical electric resistance across (Figure 2G), and they fail to express molecular markers of TJs (Figures 2 and 7). We shall now elaborate on the observations mentioned above.

Expression of Transfected β-Subunits Confers Adhesiveness to CHO Cells

It may be stressed that the transfection of CHO cells with dog β-subunit is just an experimental tool to present this subunit to MDCK cells. The fact that upon transfection with β_1-subunit CHO-β cells adjust their borders in closer contact, resembles the "epithelization" observed by Mcneill *et al.* (1990) upon transfection of E-cadherin in L-fibroblasts. Furthermore, these authors have shown that this epithelization brings about the polarization of Na^+,K^+-ATPase. Therefore, it is pertinent to point out that we have not transfected E-cadherin, nor found an endogenous E-cadherin either before nor after the expression of β_1-subunit. Rajasekaran *et al.* (2001) demonstrated that MDCK cells whose polarity was impaired by transformation with Moloney Sarcoma Virus can partially recover some of their epithelial attributes upon transfection of Na^+,K^+-ATPase β_1-subunit. This recovery is more evident when E-cadherin is cotransfected. A virus-transformed epithelial cell, however, may not be equated with a fibroblastic CHO cell, because they lack E-cadherin among other epithelial molecules. Therefore, it is not surprising that CHO cells would not express the pump in a polarized manner upon transfection of β_1-subunit.

CHO-β cells would not show signs of forming TJs either. This can be tested through the value of the electrical resistance (ER) across the cell layer. ER has two main components in parallel: the transcellular and the paracellular route. Because in most epithelia with resistances below 1.0 $K\Omega\cdot cm^2$ the resistance of the transcellular route is several orders of magnitude higher than the paracellular one, a measurement of ER across the whole cell layer only reflects the permeability of the paracellular route (Cereijido *et al.*, 1983). Such is the case of MDCK monolayers studied in the present work ($207 \pm 14\ \Omega\cdot cm^2$; Figure 2G). In turn, the ER of the paracellular route has two components arranged in series: the resistance of the tight junction and that of the intercellular space (ICS). In an epithelium with an ER above some 30 $\Omega\cdot cm^2$, the contribution of the ICS is insignificant. The ER of CHO layers is instead negligible (Figure 2G). Even when upon transfection of β-subunit ER achieves $28.3 \pm 1.5\ \Omega\cdot cm^2$ ($p < 0.001$), this relatively low value may be attributed to a narrowing of the ICS. Together, transfection of β_1-subunit, which confers adhesiveness and adoption of an "epithelial" shape of CHO-β cells, may not cause a true epithelization nor the synthesis and assembly of adherens and TJs.

β-Subunit Stabilizes the Lateral Distribution of the Sodium Pump in Epithelial Cells

Three models have been proposed for the polarized expression of Na^+,K^+-ATPase in epithelial cells. One involves intracellular sorting of newly synthesized proteins at the Golgi apparatus, followed by a vectorial delivery of the Na^+,K^+-ATPase molecules to a distinct surface domain (Caplan *et al.*, 1986; Zurzolo and Rodriguez-Boulan, 1993). Unfortunately, so far multiple efforts to identify an addressing signal in the α-subunit of this enzyme were not successful (Gottardi and Caplan, 1993; Dunbar and Caplan, 2000; Dunbar *et al.*, 2000). An alternative model emphasizes random delivery of newly synthesized Na^+,K^+-ATPase molecules to the entire plasma membrane, but selective retention of this protein at specific sites of the plasma membrane by attachment to the submembrane cytoskeleton (Nelson and Hammerton, 1989; Hammerton *et al.*, 1991). Although the association of the cytoskeleton with already polarized Na^+,K^+-ATPase has not been disputed, this model might not explain why the enzyme binds to the cytoskeleton in the membrane facing the intercellular space, and not somewhere else. In the present work, we explored the possibility that, independently of the sorting and addressing mechanisms that handle Na^+,K^+-ATPase from the *trans*-Golgi to the plasma membrane, the specific position of this enzyme is primarily due to the retention provided by an anchorage between the β-subunits in neighboring cells.

The fact that MDCK cells do not express Na^+,K^+-ATPase in heterotypic borders with NRK-E52 cells that derive from the same organ (kidney) and express the same type of isoform (β_1) suggests that the β-subunit association may be a species-specific one. Although this is in keeping with our central tenet regarding the role of the β-subunit, the specificity of the β-subunit association must await further studies using subunits derived from different animal species.

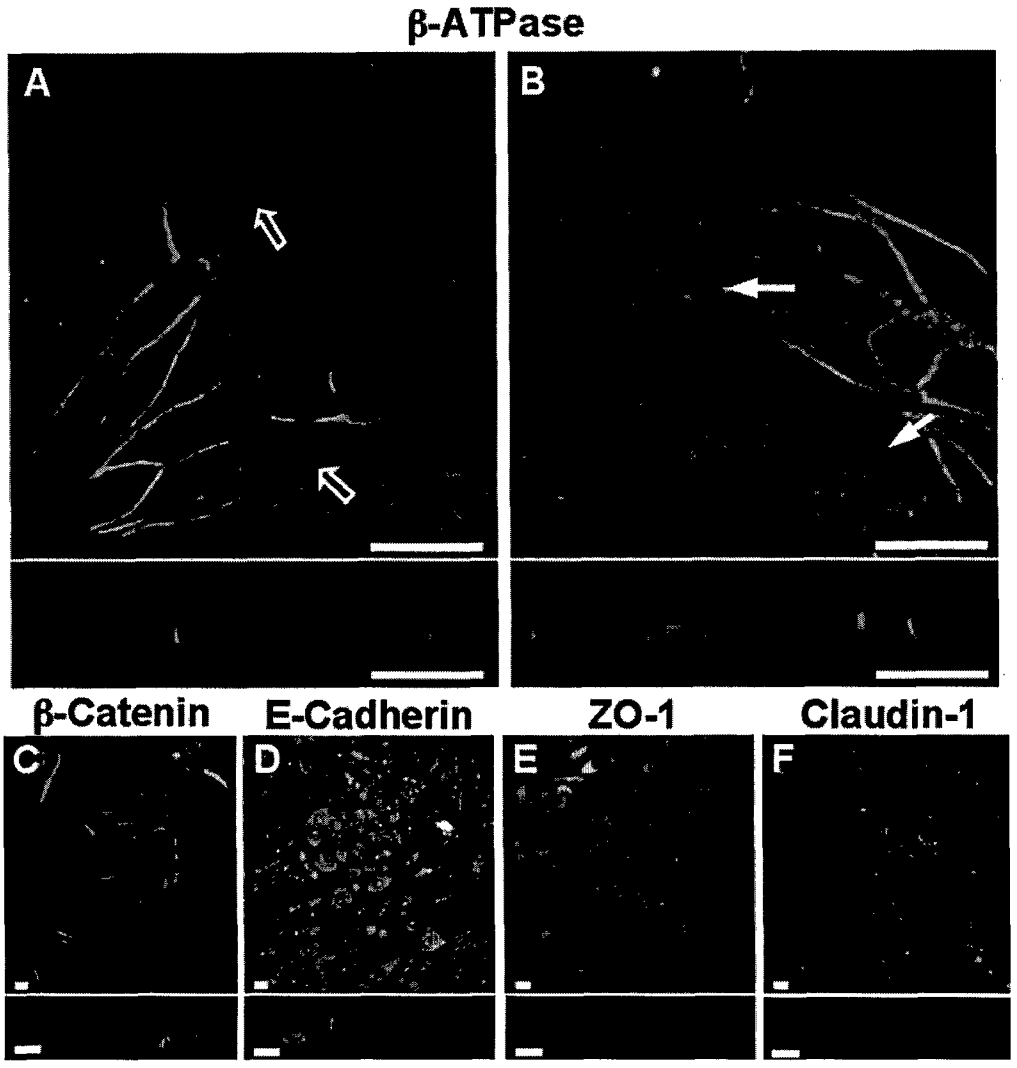

Figure 7. Expression of dog β_1-subunit in CHO cells induces MDCK cells to express their Na^+,K^+-ATPase at heterotypic borders. MDCK cells were cocultured with CHO fibroblasts (red) and assayed with antibody raised against the β_1-subunit of the dog (green). (A) MDCK cells only express the protein in homotypic MDCK/MDCK borders but not in those contacting mock-transfected CHO cells (empty arrows). (B) MDCK cocultured with CHO-β, now express their β-subunit at both homo- and heterotypic contacts (filled arrows). This induction may not be attributed to adherens nor tight junctions as CHO-β/MDCK monolayers show that β-catenin (C), E-cadherin (D), ZO-1 (E), and claudin-1 (F) are only expressed by MDCK cells.

Studies from Geering's laboratory (Geering *et al.*, 1989; Ackermann and Geering, 1990; Geering, 1990; Jaunin *et al.*, 1992) indicate that α- and β-subunits are associated from their early posttranslation steps in the endoplasmic reticulum and were never observed to be delivered to the plasma membrane separately. In agreement with these observations, and with recent work from Rajasekaran laboratory (Rajasekaran *et al.*, 2004), we show that the expression of transfected β_1-subunit from the dog in CHO-β cells induces the coexpression of hamster α-subunit (Figure 3).

Actually, the relationship between the polarized expression of the pump (P) constituted by Na^+,K^+-ATPase and the attachment (A) provided by its β-subunit was expected on three different grounds: 1) The already mentioned observation from Gloor *et al.* (Gloor *et al.*, 1990; Schmalzing *et al.*, 1992; Muller-Husmann *et al.*, 1993) that the β-subunit has the typical structure of an adhesion molecule and functions as such; 2) studies from the Takeyasu laboratory (Takeyasu *et al.*, 2001; Okamura *et al.*, 2003a,b) pointing out that early in evolution, an ancestor of this subunit was expressed independently of the α one, a circumstance that may still be present in organisms such as *Caenorhabditis elegans* and that shows that it may be involved in other functions besides of ion pumping; and 3) moreover, we have found in previous work that the occupancy of Na^+,K^+-ATPase by ouabain (Contreras *et al.*, 1999) or other molecules able to inhibit the pump (Contreras *et al.*, 2004) triggers a cascade of phosphorylations that results in retrieval of attaching molecules in the so called P→A mechanism. For further reviews of these aspects, see Cereijido *et al.* (2004).

In summary, although the α-subunit of the Na^+,K^+-ATPase accounts for most of the properties of the enzyme (ATP hydrolysis, Mg^{2+} binding, Na^+ and K^+ translocation, and ouabain binding), the β-subunit role seems to be reduced to a partner of α-subunit. Yet, in the present work, we observe that, due to the adhesiveness it confers, it may establish a linkage with a similar subunit located in a neighboring cell across the intercellular space and be responsible

for the polarized expression of Na^+,K^+-ATPase in epithelial cells.

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Molecular mechanisms mediating the anti-proliferative effects of Vitamin D in prostate cancer

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Abstract

Calcitriol (1,25-dihydroxyvitamin D_3) inhibits the growth and stimulates the differentiation of prostate cancer (PCa) cells. The effects of calcitriol are varied, appear to be cell-specific and result in growth arrest and stimulation of apoptosis. Our goal was to define the genes involved in the multiple pathways mediating the anti-proliferative effects of calcitriol in PCa. We used cDNA microarray analysis to identify calcitriol target genes involved in these pathways in both LNCaP human PCa cells and primary prostatic epithelial cells. Interestingly, two of the target genes that we identified play key roles in the metabolism of prostaglandins (PGs), which are known stimulators of PCa cell growth and progression. The expression of the PG synthesizing cyclooxygenase-2 (*COX-2*) gene was significantly decreased by calcitriol, while that of PG inactivating 15-prostaglandin dehydrogenase gene (*15-PGDH*) was increased. We postulate that this dual action of calcitriol would reduce the levels of biologically active PGs in PCa cells decreasing their proliferative stimulus and contribute to the growth inhibitory actions of calcitriol. In addition, we propose that calcitriol can be combined with non-steroidal anti-inflammatory drugs that inhibit COX activity, as a potential therapeutic strategy to improve the potency and efficacy of both drugs in the treatment of PCa.

Keywords: CYP24; CYP27B1; VDR; cDNA arrays; Target genes; Prostaglandins; 15-PGDH; COX-2; NSAIDs

1. Introduction

Prostate cancer (PCa) is the most commonly diagnosed malignancy and the second leading cause of cancer death in North American men. Primary therapy to treat PCa involves the surgical removal of the prostate or radiation therapy. However, in many men the cancer progresses to advanced or metastatic disease. Androgens play a crucial role in the development, growth and maintenance of the prostate. Most patients with metastatic PCa who have failed the primary therapy, receive drugs that block the production of androgens [1]. Although most men have a good initial response to the androgen deprivation therapy, almost all of them will eventually relapse after an average of 2–3 years. This progression develops when the cancer has evolved from androgen-dependent to androgen-independent PCa (AIPC) with limited treatment options and becomes ultimately lethal. 1,25-Dihydroxyvitamin D_3 (calcitriol), the active metabolite of Vitamin D, has emerged in recent years as a promising therapeutic agent in the treatment of PCa [2–11]. Calcitriol is an important regulator of calcium homeostasis and bone metabolism through its actions in intestine, bone, kidney and the parathyroid glands [12]. In addition to these classical actions, calcitriol also exerts anti-proliferative and pro-differentiating effects in a number of tumors and malignant cells including PCa raising the possibility of its use as an anti-cancer agent.

2. Calcitriol and prostate cancer

2.1. Epidemiological and genetic studies

PCa development has been shown to be associated with age, genetic factors and race [1]. Various studies indicate that dietary [13] and environmental factors also play a role in PCa genesis. Epidemiological data provide a strong correlation between the exposure to sunlight and the prevalence of certain cancers, particularly prostate cancer [14]. Since UV light

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is essential for Vitamin D synthesis in the skin, these studies have led to the suggestion that low circulating levels of calcitriol increase the probability of developing PCa. Moreover, polymorphisms in the gene encoding the Vitamin D receptor (VDR), which mediates the biological activity of calcitriol, may be involved in the development and or progression of PCa [2–11,13,15].

2.2. Prostate as a target for calcitriol

Many studies have demonstrated a beneficial effect of calcitriol to inhibit PCa growth and progression [2–11]. Calcitriol exerts anti-proliferative actions in several PCa cell lines [16–18], as well as in primary cultures of normal and cancer cells derived from surgical specimens taken from men with PCa [19]. The inhibition of PCa cell growth is seen in both androgen-dependent and AIPC cells [20]. Several in vivo studies on tumor xenografts established by transplanting clinical prostate tumors or cultured human PCa cells into immune-deficient mice have also demonstrated the tumor inhibitory effects of calcitriol [2–11]. The concentrations of calcitriol required for eliciting a significant growth inhibitory response in vivo causes hypercalcemia as a side effect. Therefore, investigators have used structural analogs of calcitriol that exhibit reduced calcemic effects in in vivo studies [2–7,16]. Recent findings suggest that large doses of calcitriol can be safely given to patients if administered intermittently [8,10].

3. The role of calcitriol metabolism in cellular responsiveness to the hormone

3.1. 25-Hydroxyvitamin D_3-1-α-hydroxylase (CYP27B1)

25-Hydroxyvitamin D_3-1-α-hydroxylase (1α-hydroxylase or CYP27B1) is the enzyme mediating the conversion of 25(OH)D_3 to calcitriol in the kidney, which is the major site of calcitriol synthesis [12]. Interestingly, it has been found that 1α-hydroxylase is also expressed in tissues other than the kidney including the prostate [21]. This suggests that prostate tissue is able to produce calcitriol locally and raises the possibility that hypercalcemia could be bypassed by treatment with 25(OH)D_3. However, we and others [22,23] demonstrated a substantial reduction in 1α-hydroxylase activity in human PCa cell lines and cancer derived primary prostate epithelial cells compared to the cells derived from normal prostate or benign prostatic hyperplasia (BPH). Based on these findings we speculate that administration of 25(OH)D_3 to patients is unlikely to be effective as treatment of established PCa. However, the fact that normal cells exhibit high 1α-hydroxylase activity indicates that 25(OH)D_3 may be useful as a chemopreventive agent due to its local conversion to the active hormone within the normal prostate. The cause for the decreased expression of 1α-hydroxylase in cancer cells remains to be determined.

3.2. Differential sensitivity to calcitriol action and 24-hydroxylase (CYP24) expression

24-Hydroxylase or CYP24 is the enzyme that initiates calcitriol catabolism in target cells [12]. Calcitriol induces the expression of CYP24 in target cells. Therefore, calcitriol initiates its own inactivation. CYP24 is widely expressed in calcitriol target tissues including the prostate [16]. We and others have demonstrated that the anti-proliferative action of calcitriol varies among PCa cells and is inversely related to the level of CYP24 [16,18]. For example, the DU145 PCa cells, which are resistant to the growth inhibitory action of calcitriol, exhibit a high expression of CYP24. Treatment of the DU145 cells with liarozole, an inhibitor of P450 enzymes, sensitizes the cells to the anti-proliferative actions of calcitriol [24]. Similarly, Peehl et al. showed that the combined treatment with the general P450 inhibitor, ketoconazole and calcitriol or its analog EB 1089, increased their growth inhibitory actions [25]. These results strongly suggest that the combination of calcitriol with P450 inhibitors is a useful strategy to enhance the anti-cancer activity of calcitriol in PCa treatment.

4. Clinical studies of calcitriol effects in PCa

A pilot clinical study from our lab provides evidence that calcitriol effectively slows the rate of rise of serum prostatic specific antigen (PSA) in patients with early recurrent PCa after radical prostatectomy or radiation therapy [26]. However, the amount of calcitriol administered was limited by the development of hypercalciuria. Recent advances in the design of calcitriol analogs have resulted in potential drugs with increased potency and less tendency to cause hypercalcemia [2–11]. The beneficial effects of calcitriol have been observed only at supra-physiological concentrations (>1 nmol/L). These high concentrations might be more safely achieved without causing persistent hypercalcemia if calcitriol is administered intermittently [8,10]. Recent trials using intermittent high doses of calcitriol in combination with chemotherapy drugs have shown a beneficial effect in advanced PCa [27] with calcitriol potentiating the anti-tumor effects of many cytotoxic agents [28]. Trump et al. completed a phase II study of calcitriol and dexamethasone in AIPC. This study showed a significant slowing in PSA rise in 80% of the patients with the stabilization or decrease in PSA in 34% [10]. We believe that calcitriol or a new analog will prove to be a very useful adjunct for the therapy of both androgen-dependent PCa and AIPC. It is also possible that calcitriol therapy will prove to be useful in PCa chemoprevention.

5. Molecular mechanisms mediating anti-cancer response to calcitriol in PCa

The mechanisms governing the anti-proliferative actions of calcitriol are not fully known [2–11]. A number of important pathways have been shown to have a role in

calcitriol-mediated growth inhibition. A primary mechanism of calcitriol action is to induce cell cycle arrest in the G_1/G_0 phase. The growth arrest appears to be due to an increase in the expression of cyclin-dependent kinase inhibitors $p21^{Waf/Cip1}$ and $p27^{Kip1}$, a decrease in cyclin-dependent kinase 2 (Cdk2) activity, accompanied by a reduction in the nuclear fraction of this molecule and the hyperphosphorylation of the retinoblastoma protein (pRb). In addition, calcitriol induces apoptosis in some cells and down-regulates some anti-apoptotic genes, like bcl-2. Loss of the expression of cell cycle regulators has been associated with a more aggressive cancer phenotype, decreased prognosis and poorer survival. This suggests that calcitriol may be a suitable therapy to inhibit PCa progression [2–11]. Studies from our laboratory have implicated the increased expression of insulin-like growth factor binding protein-3 (IGFBP-3) in the growth inhibition induced by calcitriol which in turn increases $p21^{Waf/Cip1}$ expression [29]. Other mechanisms of calcitriol actions in PCa cells include the stimulation of differentiation, modulation of growth factor actions and the inhibition of invasion and metastasis [2–11]. Some in vivo studies have also demonstrated that the inhibition of angiogenesis contributes to the anti-tumor effects of calcitriol [2–11].

6. Novel calcitriol target genes

We performed cDNA-microarray analyses of normal and cancer-derived primary prostatic epithelial cells and LNCaP human PCa cells [30,31] to identify the molecular targets by which calcitriol mediates its effects on PCa cells. Table 1 presents some of the genes regulated by calcitriol in prostate cells. Our studies have recently shown that calcitriol regulates the expression of the genes involved in prostaglandin (PG) metabolism which has led us to hypothesize a new pathway for the anti-cancer activity of calcitriol, namely the regulation of PG metabolism [30,31].

6.1. Calcitriol effects on prostaglandin metabolism

Our microarray analyses showed that calcitriol up-regulates the expression of NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) in LNCaP and normal primary prostate epithelial cells (see Fig. 1) [30,31]. Calcitriol also suppresses the expression of prostaglandin-endoperoxide synthase gene, also known as cyclooxygenase-2 (COX-2) in LNCaP cells (see Fig. 1). We have confirmed calcitriol regulation of these two genes by real time RT-PCR analysis. These data suggest that the regulation of PG metabolism could be an important molecular pathway of calcitriol action in prostate cells.

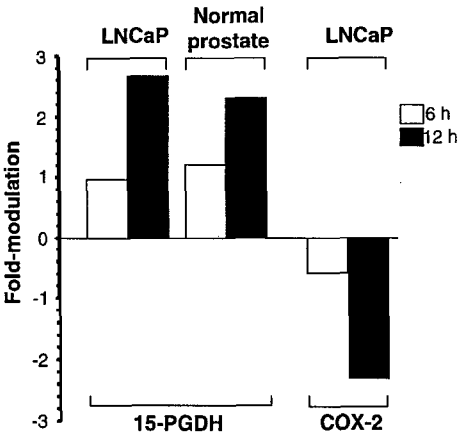

Fig. 1. Regulation of 15-PGDH and COX-2 by calcitriol in LNCaP cells and normal primary prostatic epithelial cells. LNCaP human prostate cancer cells or primary human prostatic epithelial cells derived from normal prostate were treated with calcitriol (50 nM) for 6 (□) or 24 h (■). PolyA$^+$ RNA was isolated and cDNAs were hybridized to a 24.192 element array. Data represent mean fold of increase or decrease of gene expression. (Adapted from Krishnan et al. [30] and Peehl et al. [31].)

6.1.1. COX-2

Prostaglandins play a role in the development and progression of PCa (for a review see Ref. [32]). Accumulating evidence implicates PGs as mediators of proliferation of PCa [33]. PG synthesis begins with the intracellular release of arachidonic acid (AA) from plasma membrane via the action of phospholipase A2. COX is the rate-limiting enzyme that catalyzes the conversion of AA to PGs [34]. The expression of COX-2 is rapidly induced by a variety of mitogens, cytokines, tumor promoters and growth factors, and therefore COX-2 is regarded as an immediate-early response gene [34]. Compelling evidence from genetic and clinical studies indicates that increased expression of COX-2 is one of the

Table 1
Genes modulated by calcitriol in prostate cells

Gene	Cell type	Fold change	
		6 h	24 h
Insulin-like growth factor binding protein-3	LNCaP	2.42	33.2
Vitamin D 24-hydroxylase	Normal prostate primary cells	79.1	82.5
Vitamin D 24-hydroxylase	PCa-derived epithelial cells	78.1	46.1
15-Hydroxyprostaglandin dehydrogenase	Normal prostate primary cells	1.2	2.3
15-Hydroxyprostaglandin dehydrogenase	LNCaP	0.95	2.66
Cyclooxygenase-2	LNCaP	−0.66	−2.13

Adapted from Krishnan et al. [30] and Peehl et al. [31].

key steps in carcinogenesis. Constitutive and high-levels of COX-2 activity have been detected in colon, lung, breast and prostate cancer [33,35]. It is known that high levels of PGE_2, one of the products of COX-2 activity, stimulate epithelial cell growth [33], promote cell survival and invasion [36]. Several studies have demonstrated COX-2 over-expression in prostate adenocarcinoma [32,37] suggesting a positive role for COX-2 in prostate tumorigenesis. COX-2 expression appears to be linked to cell survival. For example, COX-2 over-expression leads to the stabilization of survivin [38], a member of the inhibitor of apoptosis protein family that block caspase activation. The stabilization of survivin alters the balance between pro- and anti-apoptotic pathways leading to resistance to apoptosis. COX-2 expression has also been associated with angiogenesis and tumor metastasis [37] and the administration of a COX-2 inhibitor suppresses the growth of prostate tumor xenograft in mice by inhibiting angiogenesis [32]. We have found that calcitriol treatment decreases COX-2 gene expression in PCa cells (Fig. 1) [30]. Our data suggest that COX-2 suppression by calcitriol might contribute to the hormone's tumor inhibitory actions and anti-metastatic potential.

6.1.2. 15-PGDH

15-PGDH catalyzes the conversion of PGs into mostly inactive 15-keto derivatives [39]. A growing body of evidence has implicated 15-PGDH as a potential target for cancer therapy [40,41]. Various studies indicate that 15-PGDH acts as a putative tumor suppressor gene in lung cancer [41]. Backlund et al. [42] demonstrated a down-regulation of 15-PGDH expression in colorectal carcinoma. 15-PGDH has recently been described as an oncogene antagonist that functions as a tumor suppressor in colon cancer [43]. The study found that 15-PGDH, which physiologically antagonizes COX-2, was universally expressed in normal colon specimens but was routinely absent or severely reduced in cancer specimens. The study also showed that 15-PGDH expression was induced by transforming growth factor β (TGFβ) and that colon tumor cells exhibited mutations in TGFβ receptors or the genes involved in the SMAD signaling pathway explaining the decreased 15-PGDH levels in colon cancer. Most importantly, stable transfection of a 15-PGDH expression vector into cancer cells greatly reduced the ability of the cells to form tumors and/or slowed tumor growth in nude mice. The authors concluded that 15-PGDH suppressed the effects of the oncogene COX-2 and had an additional effect to inhibit angiogenesis in vivo [43]. Our studies show an up-regulation of 15-PGDH gene expression by calcitriol in normal and malignant prostate cells (Fig. 1) [30,31] and this regulation might mediate some of the anti-cancer effects of calcitriol. Interestingly, calcitriol has also been shown to induce the expression of 15-PGDH in human neonatal monocytes [44].

Based on our observations summarized in Table 1, we hypothesize that calcitriol treatment of PCa cells would reduce the levels of biologically active PGs due to its dual actions to reduce COX-2 expression and increase 15-PGDH expression. We further propose that the modulation of PG metabolism is an important molecular mechanism mediating the anti-proliferative and anti-metastatic activities of calcitriol.

7. Calcitriol and NSAIDs

Recent exciting findings show that non-steroidal anti-inflammatory drugs (NSAIDs) exert chemopreventive effects in several cancers [32,45]. NSAIDs have also been shown to suppress PCa development and progression [32,46]. The primary action of NSAIDs is to inhibit PG synthesis by directly inhibiting the enzymatic activity of COX-1 as well as COX-2. Many in vitro and in vivo studies have demonstrated that NSAIDs inhibit PCa growth and cause apoptosis of PCa cells [32,45]. However, many NSAIDs exhibit differences between their ability to inhibit COX-2 and to induce apoptosis suggesting that apoptosis induction may be independent of COX-2 inhibition [47]. The actions of NSAIDs on 15-PGDH are not clear. While many NSAIDs have been shown to inhibit the enzymatic activity of 15-PGDH [48], the non-selective NSAID indomethacin has been reported to enhance the expression and activity of 15-PGDH in thyroid carcinoma [49].

We hypothesize that the combination of calcitriol and NSAIDs would be additive/synergistic in their activity to inhibit PCa growth. The action of calcitriol at the genomic level to suppress COX-2 gene expression will decrease the levels of COX-2 protein and allow the use of lower concentrations of NSAIDs to inhibit COX-2 enzyme activity. The action of calcitriol to increase 15-PGDH expression would also complement NSAID action. The use of COX-2 selective NSAIDs has recently been shown to cause some serious cardiovascular side effects, such as increased risk of heart attacks, stroke, sudden death and blood clots [50]. The undesirable effect of calcitriol therapy is limited to hypercalcemia. We propose that a combination therapy of calcitriol with a NSAID would allow the use of lower concentrations of both drugs, thus reducing their individual side effects while increasing their anti-proliferative and pro-apoptotic activities.

8. Conclusions

Our research is directed at understanding the molecular mechanisms of the anti-proliferative activity of calcitriol in prostate cells with the goal of developing strategies to improve PCa treatment. Using cDNA microarrays we have recently found that calcitriol modulates the expression genes involved in PG metabolism. Calcitriol suppresses the expression of COX-2 gene, the enzyme that catalyzes PG synthesis and up-regulates the expression of 15-PGDH gene, the enzyme involved in PG inactivation. We hypothesize that

calcitriol treatment of PCa cells would reduce the levels of biologically active PGs due to its dual actions on COX-2 and 15-PGDH expression, and thereby decrease the PG-mediated proliferative stimulus. We further propose that the calcitriol effects would complement the action of NSAIDs, which are known inhibitors of both COX-1 and COX-2. The action of calcitriol at the genomic level to suppress COX-2 gene expression will decrease the levels of COX-2 protein and allow the use of lower the concentrations of NSAIDs needed to inhibit COX-2 enzyme activity. A combination of calcitriol with a NSAID would allow the use of lower concentrations of both drugs, thus reducing their individual side effects while increasing their anti-proliferative and pro-apoptotic activities. The combination approach is an attractive therapeutic strategy in the treatment of PCa and can be brought to clinical trials swiftly.

Note added in proof

Since this paper was originally written we have used real-time PCR and Western blot analyses to confirm that calcitriol inhibits PG actions in human PCa cell lines and primary prostatic epithelial cells. We found that calcitriol increased the expression of 15-PGDH, reduced the expression of COX-2, and reduced the expression of EP2 and FP PG receptors. These three calcitriol actions combine to decrease secreted PGE_2 and block cell growth stimulated by arachidonic acid and exogenous PGs. Moreover, calcitriol, in combination with various NSAIDs, produces synergistic inhibition of PCa cell growth at lower and safer drug concentrations. These data suggest that calcitriol and NSAIDs may be a useful combination for chemotherapy and/or chemoprevention of PCa. These new findings are now in press in Cancer Research 2005.

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Regulation of Prostaglandin Metabolism by Calcitriol Attenuates Growth Stimulation in Prostate Cancer Cells

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Abstract

Calcitriol exhibits antiproliferative and prodifferentiation effects in prostate cancer. Our goal is to further define the mechanisms underlying these actions. We studied established human prostate cancer cell lines and primary prostatic epithelial cells and showed that calcitriol regulated the expression of genes involved in the metabolism of prostaglandins (PGs), known stimulators of prostate cell growth. Calcitriol significantly repressed the mRNA and protein expression of prostaglandin endoperoxide synthase/cyclooxygenase-2 (COX-2), the key PG synthesis enzyme. Calcitriol also up-regulated the expression of 15-hydroxyprostaglandin dehydrogenase, the enzyme initiating PG catabolism. This dual action was associated with decreased prostaglandin E_2 secretion into the conditioned media of prostate cancer cells exposed to calcitriol. Calcitriol also repressed the mRNA expression of the PG receptors EP2 and FP, providing a potential additional mechanism of suppression of the biological activity of PGs. Calcitriol treatment attenuated PG-mediated functional responses, including the stimulation of prostate cancer cell growth. The combination of calcitriol with nonsteroidal anti-inflammatory drugs (NSAIDs) synergistically acted to achieve significant prostate cancer cell growth inhibition at ~2 to 10 times lower concentrations of the drugs than when used alone. In conclusion, the regulation of PG metabolism and biological actions constitutes a novel pathway of calcitriol action that may contribute to its antiproliferative effects in prostate cells. We propose that a combination of calcitriol and nonselective NSAIDs might be a useful chemopreventive and/or therapeutic strategy in men with prostate cancer, as it would allow the use of lower concentrations of both drugs, thereby reducing their toxic side effects. (Cancer Res 2005; 65(17): 7917-25)

Introduction

In the United States, prostate cancer remains the most common solid tumor malignancy in men, causing ~30,000 deaths in 2005 (1). Effective treatment options include surgery and radiation therapy. The main treatment strategy for advanced prostate cancer involves androgen deprivation therapy to which patients initially respond very well. However, most patients eventually fail this therapy and frequently develop metastatic disease. Current research on prostate cancer aims to identify new agents that would prevent and/or inhibit its progression.

1,25-Dihydroxyvitamin D_3 (calcitriol), the active form of vitamin D, is the major regulator of calcium and phosphate homeostasis in bone, kidney, and intestine (2). However, calcitriol has also been shown to exhibit antiproliferative and prodifferentiation effects in many normal and malignant cells including prostate cancer cells (3–10). There are multiple mechanisms underlying the antiproliferative effects of calcitriol, which vary between target cells (10). These include cell cycle arrest (9, 11) and the induction of apoptosis (12). Several genes that mediate these growth regulatory effects have been identified to be the molecular targets of calcitriol action, such as *p21, p27, bcl-2,* and insulin-like growth factor binding protein-3 (*IGFBP-3*; refs. 5–14). We recently did cDNA microarray analyses to more fully characterize the spectrum of genes regulated by calcitriol in prostate cells (15, 16). Among the newly identified genes regulated by calcitriol, we found two genes which play a key role in prostaglandin (PG) metabolism: the prostaglandin endoperoxide synthase-2 or cyclooxygenase (COX)-2 and the NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH). PGs are synthesized from free arachidonic acid (17) by COXs. There are two well-characterized COX isoforms: COX-1, a constitutive form of the enzyme, and COX-2, an inducible form of the enzyme. PGs are implicated in the initiation and progression of many malignancies including prostate cancer (18–20). Tumor cells with elevated COX-2 levels are highly resistant to apoptosis, show increased angiogenic potential, and exert suppressive effects on host immunity (19, 20). Nonsteroidal anti-inflammatory drugs (NSAIDs), known inhibitors of both COX-1 and COX-2 enzymatic activity, are under intense investigation to prevent and/or treat malignancies (19, 21). 15-PGDH, which mediates the catalytic inactivation of PGs by converting them to the corresponding keto derivatives, has been found to be down-regulated in some cancers (22, 23) and has recently been regarded as a tumor suppressor gene (24).

In the current study, we investigated the regulation of COX-2 and 15-PGDH by calcitriol in the androgen-dependent LNCaP and androgen-independent PC-3 human prostate cancer cell lines as well as in primary prostatic epithelial cells derived from normal and cancerous human prostate tissue. Calcitriol reduced the expression of COX-2 and increased that of 15-PGDH. Calcitriol treatment of prostate cancer cells decreased the concentration of prostaglandin E_2 (PGE_2) secreted into the conditioned medium. In addition, calcitriol also decreased the expression of the mRNA of PG receptors EP2 and FP. Our data indicate that these mechanisms led to the attenuation of PG-mediated functional responses by calcitriol, including the suppression of PG stimulation of cell growth. Further, our study showed that the combination of calcitriol and NSAIDs exhibited synergistic growth inhibition, suggesting that the combination might be a useful therapeutic and/or chemopreventive strategy in prostate cancer.

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Materials and Methods

Materials

PGE_2, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), arachidonic acid, NS-398, and SC-58125 were obtained from Cayman Chemical Co. (Ann Arbor, MI). Calcitriol was a gift from Leo Pharma A/S (Ballerup, Denmark). All stock solutions were made in 100% ethanol and stored at −20°C. Tissue culture media were obtained from Mediatech, Inc. (Herndon, VA). Tissue culture supplements and fetal bovine serum (FBS) were obtained from Life Technologies, Inc. (Grand Island, NY).

Methods

Cell culture. LNCaP (ATCC no. CRL-1740) and PC-3 (ATCC no. CRL-1435) cells were grown in RPMI 1640 supplemented with 5% FBS, 100 IU/mL of penicillin, and 100 μg/mL streptomycin (Life Technologies). Cells were maintained at 37°C with 5% CO_2 in a humidified incubator. Primary cells were derived from radical prostatectomy specimens from men undergoing surgery for prostate cancer treatment. None of the patients had received prior therapy for prostate cancer. The normal cell strains (E-PZ-1 to -3) were derived from peripheral zone tissue with no histologic evidence of cancer in adjacent sections. The cancer cell strains used, E-CA-1 (Gleason grade 3/3), E-CA-2 (Gleason grade 4/5), and E-CA-3 (Gleason grade 4/3), were derived from adenocarcinoma specimens. Primary cell cultures were established from the prostate tissue samples and propagated in culture as we have previously described (25).

Cell proliferation assay. Prostate cancer cells were seeded at an initial density of 1.5×10^5 cells/well in six-well tissue culture plates and allowed to attach overnight in RPMI 1640 with 5% FBS. Cell cultures were shifted to medium containing 2% FBS and treated in triplicate over the next 6 days with either 0.1% ethanol vehicle or the indicated concentrations of drugs. Fresh media and the drugs were replenished every other day. At the end of the treatment, the cells were collected by gentle scraping and subjected to lysis in 0.2 N NaOH. Cell proliferation was assessed by the determination of DNA content (26).

RNA isolation and real-time reverse transcription-PCR. Total RNA was isolated from vehicle or drug-treated cells by the Chomczynski method using Trizol reagent (Invitrogen, Life Technologies, Inc., Carlsbad, CA) as previously described (15). The yield and purity of isolated RNA were checked by UV spectrophotometry. Five micrograms of total RNA were used in reverse transcription reactions using the SuperScript III first strand synthesis kit (Invitrogen). Gene expression in vehicle or calcitriol-treated cells was determined by real-time PCR using the reverse transcription product and gene-specific primers. The reactions were carried out with the DyNamo SYBR Green qPCR kit (Finnzymes, Oy, M.J. Research, Reno, NV) in a 20 μL reaction volume containing gene-specific primers (10 pmol). All real-time PCR reactions were done in duplicate according to the following program: incubation at 72°C for 5 minutes, incubation at 95°C for 5 minutes, and 40 cycles of 94°C for 20 seconds, 58°C for 15 seconds, and 72°C for 20 seconds. PCR products were subjected to agarose gel electrophoresis to determine the purity and size of the amplified products (27). Real-time PCR was carried out using an Opticon 2 DNA engine (M.J. Research). Relative changes in mRNA expression levels were assessed by the $2^{-\Delta\Delta C(T)}$ method (28). Changes in mRNA expression of the different genes were normalized to that of TATA binding protein (*TBP*) gene or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene. The primers used were as follows: COX-2: 5′-GATACTCAGGCAGAGATGATCTACCC-3′ (sense), 5′-AGACCAGGCACCAGACCAAAGA-3′ (antisense); 15-PGDH: 5′-GACTCTGTTCATCCAGTGCG-3′ (sense), 5′-CCTTCACCTCCATTTTGCTTACTC-3′ (antisense); *c-fos*: 5′-GAATAAGATGGCTGCAGCCAAATGCCGCAA-3′ (sense), 5′-CAGTCAGATCAAGGGAAGCCACAGACATCT-3′ (antisense; ref. 29); EP2: 5′-GTGCTGACAAGGCACTTCATGT-3′ (sense), 5′-TGTTCCTCCAAAGGCCAAGTAC-3′ (antisense); FP: 5′-GCACATTGATGGGCAACTAGAA-3′ (sense), 5′-GCACCTATCATTGGCATGTAGCT-3′ (antisense); *TBP*: 5′-CACTCACAGACTCTCACAACTGC-3′ (sense), 5′-GTGGTTCGTGGCTCTCTTATC-3′ (antisense); *GAPDH*: 5′-GCCTCAAGATCATCAGCA-3′ (sense), 5′-GTTGCTGTAGCCAAATTC-3′ (antisense).

Measurement of prostaglandin E_2 secretion. Subconfluent LNCaP cells were treated with vehicle or calcitriol for 48 hours. Conditioned media were collected and secreted PGE_2 levels were quantitated using a PGE_2 monoclonal enzyme immunoassay kit (Cayman Chemical) according to the protocol of the manufacturer.

Western blot analysis. Cell lysates were prepared from vehicle or calcitriol-treated cells by lysis with a buffer containing 50 mmol/L Tris-HCl, 1 mmol/L EDTA, and 1.6 mmol/L CHAPS (Sigma-Aldrich, St. Louis, MO) supplemented with a protease inhibitor cocktail (Complet, Roche Diagnostics GmbH, Mannheim, Germany). Lysates were incubated at 4°C for 20 minutes and centrifuged at 10,000 × *g* for 1 minute to sediment particulate material. The protein concentration of the supernatant was measured by the Bradford method (30). Proteins were separated in either 10% NuPAGE gels in MOPS-SDS running buffer (Invitrogen) or 10% polyacrylamide Tris-Tricine (Sigma-Aldrich) gels depending on the size of the protein to be detected. After electrophoresis, proteins were transferred onto nitrocellulose membranes by electroblotting. The COX-2 monoclonal (1:1000 dilution) and 15-PGDH polyclonal antibodies (1:250 dilution) used in our study were purchased from Cayman Chemicals. β-Actin monoclonal antibody (dilution 1:500) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Membranes were incubated with the appropriate primary antibodies followed by incubations with a secondary antibody to immunoglobulin G conjugated to horseradish peroxidase (Cell Signaling Technology, Inc., Beverly, MA). Immunoreactive bands were visualized using the enhanced chemiluminescence Western blot detection system (Amersham, Piscataway, NJ) according to the instructions of the manufacturer. The blots were also probed for the expression of β-actin as a control. COX-2 protein was visualized as a ~70 kDa immunoreactive band. 15-PGDH protein was visualized as a ~29 kDa immunoreactive band. Immunoreactive bands were scanned by densitometry (HP Scanjet 7400C) and quantified using Bio-Rad software (Bio-Rad, Hercules, CA). COX-2 or 15-PGDH signals were normalized to β-actin levels in each sample.

Results

We previously showed by cDNA microarray analysis that calcitriol regulated the expression of two of the key genes involved in PG metabolism (i.e., COX-2 and 15-PGDH) in LNCaP human prostate cancer cells (15) and 15-PGDH in primary normal prostatic epithelial cells (16). In the present study, we extended these observations to include an evaluation of calcitriol effects on the expression of these two genes at both the mRNA and protein levels in LNCaP and PC-3 cells. In addition, we also examined the effects of calcitriol in primary prostatic epithelial cell strains derived from normal prostate as well as prostate adenocarcinoma specimens.

Down-regulation of cyclooxygenase-2 expression by calcitriol. Real-time reverse transcription-PCR (RT-PCR) analysis showed significant decreases in COX-2 mRNA levels in both androgen-dependent LNCaP (~70% inhibition) and androgen-independent PC-3 cells (~45% inhibition) due to calcitriol treatment (Fig. 1*A*). Although both LNCaP and PC-3 prostate cancer cells have been shown to express COX-2 protein (31), we found that PC-3 cells exhibited higher basal levels of COX-2 protein expression when compared with LNCaP cells (not shown). We therefore used PC-3 cells to assess the effect of calcitriol on COX-2 protein expression. Figure 1*B* shows that the addition of 10 nmol/L calcitriol to PC-3 cultures for 48 hours reduced COX-2 protein level to ~60% of control, with 100 nmol/L calcitriol having no further effect.

Up-regulation of 15-hydroxyprostaglandin dehydrogenase expression by calcitriol. We examined the effect of calcitriol on 15-PGDH mRNA levels in LNCaP and PC-3 cells. Our data show that 10 nmol/L calcitriol increased 15-PGDH mRNA expression by ~3.6-fold in LNCaP cells and by ~3-fold in PC-3 cells (Fig. 1*C*). We found that the basal protein expression of 15-PGDH varied between different cell lines with the LNCaP exhibiting appreciable levels of the 15-PGDH protein whereas barely detectable levels were seen in PC-3 cells. Therefore, we examined the effect of

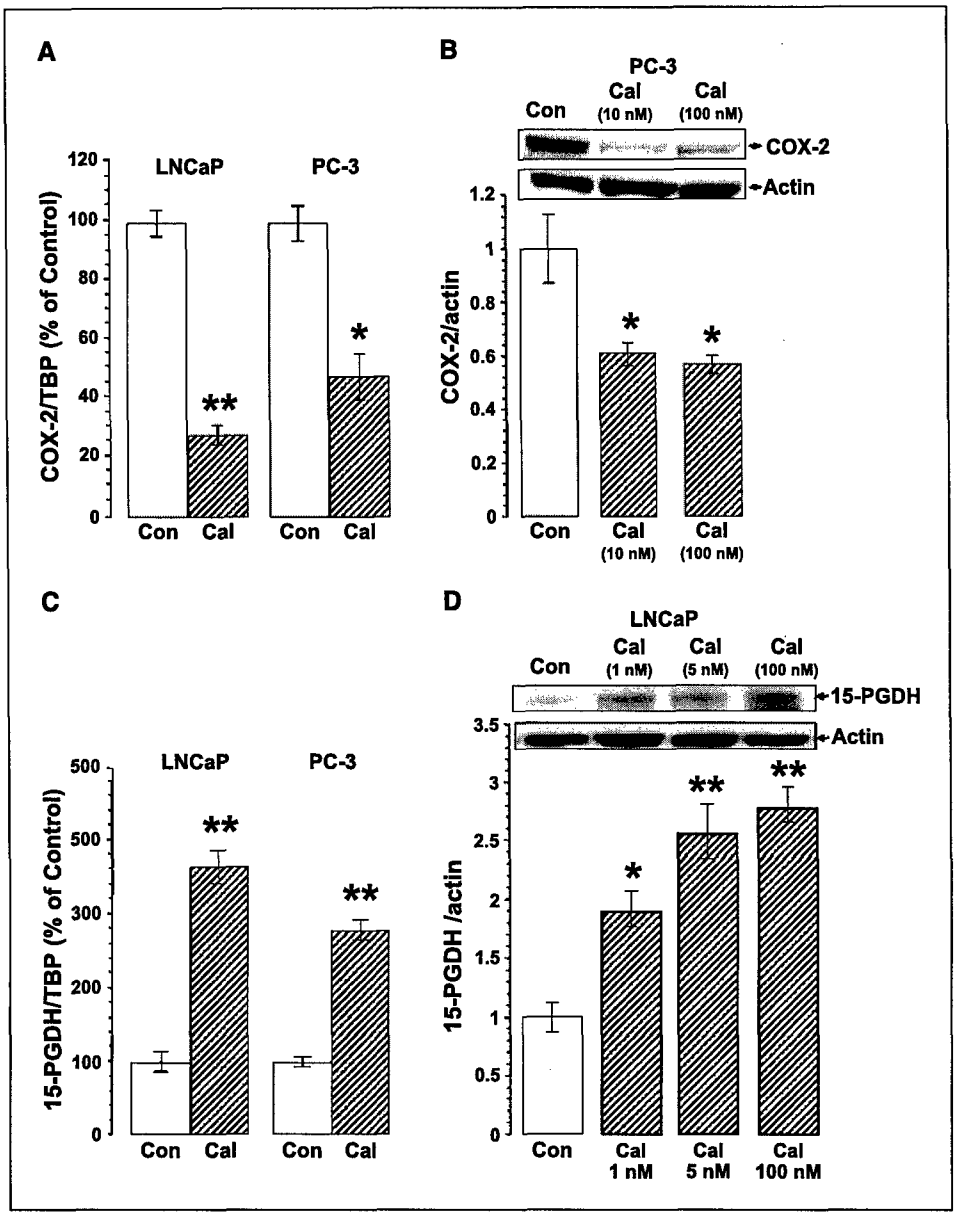

Figure 1. Calcitriol regulates COX-2 and 15-PGDH expression in prostate cancer cell lines. *A*, calcitriol decreases COX-2 mRNA levels. Subconfluent cultures of LNCaP and PC-3 cells were treated with 0.1% ethanol (*Con*) or 10 nmol/L calcitriol (*Cal*) for 24 hours and total RNA was extracted. COX-2 mRNA levels were determined by real-time RT-PCR as described in Materials and Methods and were normalized to *TBP* mRNA levels in the same samples. COX-2/TBP ratio shown as a percent of control set at 100%; *columns*, mean from five experiments; *bars*, SE. *B*, calcitriol decreases COX-2 protein levels. Subconfluent cultures of PC-3 cells were treated with 0.1% ethanol (*Con*) or 10 or 100 nmol/L calcitriol (*Cal*) for 48 hours. Fifty micrograms of total protein were subjected to Western blot analysis as described in Materials and Methods. Representative Western blot. The densitometric units of COX-2 immunoreactive bands were normalized to the densitometric units of the corresponding β-actin bands. Results expressed as the ratio of the control set at 1. *C*, calcitriol increases 15-PGDH mRNA levels. Cells were treated and processed as described in *A*. 15-PGDH/TBP ratio in calcitriol-treated cells given as a percent of control set at 100%; *columns*, mean from five experiments; *bars*, SE. *D*, calcitriol increases 15-PGDH protein levels. LNCaP cells were treated as in *B*. The densitometric units of 15-PGDH immunoreactive bands were normalized to the densitometric units of the corresponding β-actin bands. Results expressed as the ratio of the control set at 1; *columns*, mean of three experiments; *bars*, SE. *, $P < 0.05$; **, $P < 0.01$, when compared with control.

calcitriol on 15-PGDH protein expression in LNCaP cells and found a dose-dependent increase in 15-PGDH protein levels in response to calcitriol treatment (Fig. 1*D*).

Calcitriol effects on cyclooxygenase-2 and 15-hydroxyprostaglandin dehydrogenase mRNA levels in primary prostatic epithelial cells. We extended our analysis to include calcitriol effects on primary cultures of prostatic epithelial cells derived from normal prostate as well as adenocarcinoma specimens removed at surgery. Real-time RT-PCR analysis showed considerable decreases (55-90%) in COX-2 mRNA levels in two of the three normal primary cell strains tested (E-PZ-1 and E-PZ-3) after 24 hours of calcitriol treatment (Fig. 2*A*). In all three cancer-derived primary cultures (E-CA-1 to -3) significant reductions (~48-60%) in COX-2 mRNA levels were seen at an earlier time point, after 6 hours of calcitriol treatment, and the down-regulatory effect was lost by 24 hours except in the case of E-CA-2 (Fig. 2*B*). Figure 2*C* and *D* shows the calcitriol-induced changes in 15-PGDH mRNA in primary prostatic cells. In the normal primary cells, calcitriol treatment caused appreciable increases in 15-PGDH mRNA in two of the three strains tested. The time course of this effect showed minor differences. In E-PZ-1 and E-PZ-2 cells significant increases (~2- to 18-fold) were achieved at the end of 6 and 24 hours, respectively (Fig. 2*C*). In two of three of the cancer-derived primary cultures (E-CA-2 and -3), significant increases (~2- to 5-fold) were seen at the end of 24 hours (Fig. 2*D*). In general, the magnitude of COX-2 mRNA down-regulation as well as 15-PGDH mRNA increase was more pronounced in the primary cells derived from normal prostatic tissue when compared with both the cancer-derived primary cells and the established prostate cancer cell lines.

Effect of calcitriol on prostaglandin levels. As a result of the dual action of calcitriol to down-regulate the expression of PG synthesizing COX-2 and increase the PG catabolizing 15-PGDH, we expected a reduction in PG production and secretion in prostate cancer cells treated with calcitriol. We measured the levels of PGE_2 in the conditioned media from LNCaP cells treated with various concentrations of calcitriol for 48 hours. Figure 3*A* shows that the

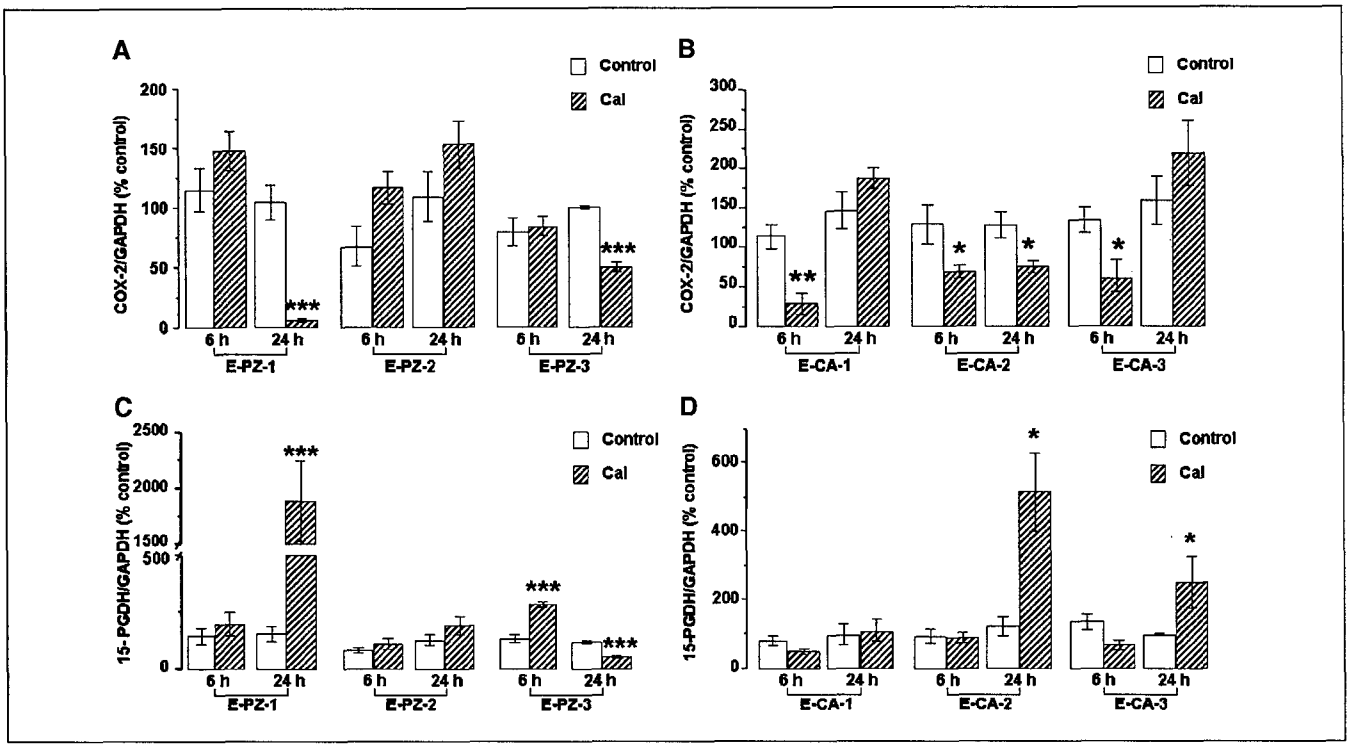

Figure 2. Calcitriol regulates the expression of COX-2 and 15-PGDH mRNA in primary prostatic epithelial cells. Primary cultures of prostatic epithelial cells derived from the peripheral zone of normal prostate tissue (E-PZ-1 to -3) or adenocarcinoma (E-CA-1 to -3) were treated with 0.1% ethanol (*Con*) or with 10 nmol/L calcitriol (*Cal*) for 6 or 24 hours. Total RNA was extracted and COX-2 and 15-PGDH mRNA levels were quantitated by real-time RT-PCR using gene-specific primers as described in Materials and Methods. COX-2 and 15-PGDH mRNA levels were normalized to *GAPDH* mRNA levels and are given as a percent of control set at 100%. *Columns,* mean from three experiments; *bars,* SE. Effect of calcitriol on COX-2 mRNA in three different normal primary epithelial cell strains (*A*) and in cancer-derived cell strains (*B*). Changes in 15-PGDH mRNA in normal cell strains (*C*) and in cancer-derived cell strains (*D*). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, when compared with control.

addition of calcitriol caused a significant reduction in PGE_2 secretion with the maximal decrease (~34%) seen with 100 nmol/L calcitriol.

Effects of calcitriol on prostaglandin receptor expression. Prostate cancer cells have been shown to express the PGE receptor subtypes EP2 and EP4 (29). We examined the effects of calcitriol on the expression of the PGE_2 receptor isoforms EP1, EP2, EP3, and EP4, and the $PGF_{2\alpha}$ receptor FP. LNCaP cells treated with 10 nmol/L calcitriol for 24 hours showed a significant (~45%) down-regulation of EP2 mRNA (Fig. 3*B*). We did not detect any changes in the levels of EP1, EP3, or EP4 mRNA following calcitriol treatment (not shown). FP mRNA levels were also down-regulated (~33% decrease) by calcitriol (Fig. 3*C*).

Inhibition of prostaglandin-mediated induction of *c-fos* mRNA by calcitriol. Because calcitriol modulated the levels of biologically active PGs as well as PG receptor expression, we examined its effect on a PG-mediated functional response, (i.e., the induction of the immediate-early gene *c-fos*; ref. 29). As serum is a potent inducer of *c-fos* expression (32), we conducted the experiment under serum-free conditions using PC-3 cells. Unlike LNCaP, PC-3 cells could be briefly maintained in serum-free media for calcitriol pretreatment and subsequent treatment with the PG precursor arachidonic acid. PC-3 cells were pretreated with vehicle or 10 nmol/L calcitriol for 48 hours followed by a brief (30 minutes) exposure to exogenous arachidonic acid (3 μmol/L) directly added to the culture medium. RNA was then isolated and the induction of *c-fos* mRNA was determined as an indicator of the biological activity of PGs endogenously synthesized from arachidonic acid. As shown in Fig. 3*D*, in vehicle pretreated cells arachidonic acid exposure resulted in a significant induction (~2.5-fold) of *c-fos* mRNA levels after 30 minutes. Calcitriol pretreatment completely abrogated the induction of *c-fos* mRNA due to arachidonic acid addition. Calcitriol pretreatment by itself caused a minor increase in *c-fos* mRNA levels when compared with vehicle pretreated cells, which was not statistically significant.

Effects of calcitriol on prostaglandin-mediated growth stimulation. We examined the effect of calcitriol on the stimulation of prostate cancer cell growth by exogenous PG addition as well as by endogenous PGs derived from the substrate arachidonic acid added to the culture medium. We treated LNCaP and PC-3 cells with arachidonic acid (3 μmol/L), PGE_2, or $PGF_{2\alpha}$ (10 μmol/L each) in the absence or presence of 10 nmol/L calcitriol. Our results revealed a moderate but significant growth stimulation by arachidonic acid and exogenous PGs in both LNCaP (Fig. 4*A*) and PC-3 cells (Fig. 4*B*). Calcitriol had a marked growth inhibitory action when given alone. In addition, calcitriol blocked the growth stimulation due to endogenous PGs derived from the added arachidonic acid as well as exogenous PG addition (Fig. 4*A* and *B*).

Synergistic inhibition of prostate cancer cell growth by calcitriol and nonsteroidal anti-inflammatory drugs. We next examined the combined effect of calcitriol and NSAIDs, which are potent inhibitors of COX enzyme activity. We tested a number of both COX-2–selective and nonselective NSAIDs including NS-398, SC-58125, flufenamic acid, sulindac sulfide, indomethacin, naproxen, and ibuprofen. Figure 5*A* to *D* illustrates the effect on prostate cancer cell growth of calcitriol alone

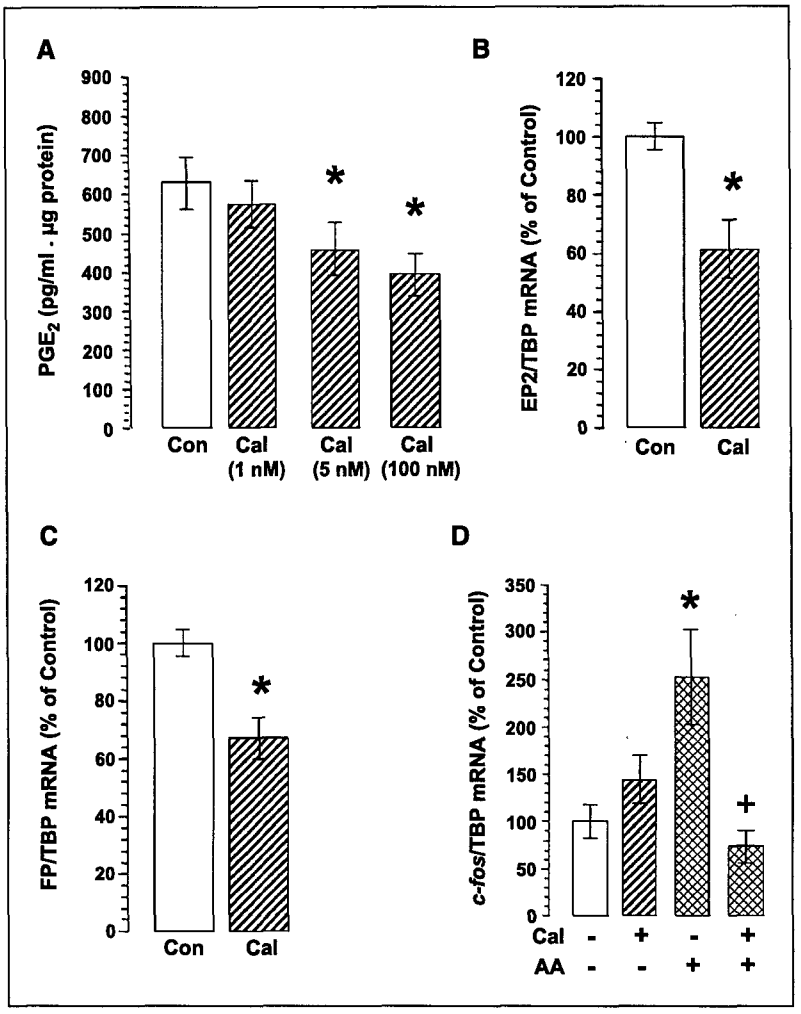

Figure 3. *A*, calcitriol decreases PGE_2 levels. Subconfluent cultures of LNCaP cells were treated with 0.1% ethanol (*Con*) or with the indicated concentrations of calcitriol (*Cal*) for 48 hours. Conditioned media from control and calcitriol-treated cultures were collected and PGE_2 levels were determined using an enzyme immunoassay kit (Materials and Methods). *Columns*, mean from three experiments; *bars*, SE. *, $P < 0.05$. *B*, changes due to calcitriol treatment in EP2 mRNA. LNCaP cells were grown to subconfluence and treated with vehicle (0.1% ethanol; *Con*) or 10 nmol/L calcitriol (*Cal*) for 24 hours. Total RNA was extracted and analyzed for the mRNA expression of EP2 by real-time RT-PCR using gene-specific primers as described in Materials and Methods. EP2 mRNA levels were normalized to the *TBP* mRNA levels. Values given as a percent of control set at 100%; *columns*, mean from three experiments; *bars*, SE. *, $P < 0.05$, when compared with control. *C*, changes due to calcitriol treatment in FP mRNA. LNCaP cells were treated and processed as in *B* for the mRNA expression of FP by real-time RT-PCR. FP mRNA levels were normalized to the *TBP* mRNA levels. Values are given as a percent of control set at 100%; *columns*, mean from three experiments; *bars*, SE. *, $P < 0.05$, when compared with control. *D*, calcitriol inhibits PG-mediated induction of *c-fos* mRNA. Subconfluent cultures of PC-3 cells were transferred to serum-free RPMI 1640 containing 0.1% ethanol vehicle or 10 nmol/L calcitriol during 48 hours (pretreatment). Following the pretreatment, the cultures were exposed for 30 minutes to arachidonic acid (*AA*; 3 µmol/L) added to the culture medium. The cell cultures were then scraped, RNA was isolated, and *c-fos* mRNA levels were determined by real-time RT-PCR as described in Materials and Methods. *c-fos* mRNA levels were normalized to *TBP* mRNA levels and are given as a percent of control set at 100%. *Columns*, mean from three experiments; *bars*, SE. *, $P < 0.05$, when compared with control; +, $P < 0.05$, when compared with arachidonic acid.

or in combination with the NSAIDs that exhibited the best growth inhibitory effect when used at a reduced dose. We show the effects of calcitriol alone and in combination with the COX-2–selective NSAIDs SC-58125 on the growth of LNCaP cells (Fig. 5*A*) and NS-398 on the growth of PC-3 cells (Fig. 5*B*). In LNCaP cells, calcitriol by itself had a modest effect (~20%) at 1 nmol/L but caused significant growth inhibition (~40%) at 10 nmol/L (Fig. 5*A*). The addition of the COX-2–specific inhibitor SC-58125 by itself had a modest effect on cell growth (~20% inhibition), which was not statistically significant, at the concentration tested (5 µmol/L). The combination of 1 nmol/L calcitriol with SC-58125, however, had a more pronounced inhibitory effect (~73% growth inhibition with the combination versus ~20% inhibition with the individual agents), indicating a synergistic interaction between these two drugs to inhibit cell growth. SC-58125 also enhanced the growth inhibition seen with the higher concentration of calcitriol (~80% inhibition with the combination versus ~40% inhibition with 10 nmol/L calcitriol alone). Similar synergistic growth inhibitory effects were evident in PC-3 cells treated with a combination of calcitriol and the COX-2–selective inhibitor NS-398 (Fig. 5*B*). NS-398, when used alone at 7.5 µmol/L, did not affect the growth of PC-3 cells. However, it enhanced the growth inhibition seen with both 1 and 10 nmol/L calcitriol (~60% inhibition with the combination versus ~20% inhibition with 1 nmol/L calcitriol alone, and ~75% inhibition with the combination versus ~40% inhibition with 10 nmol/L calcitriol alone).

The growth inhibitory effect of calcitriol was similarly enhanced when combined with nonselective NSAIDs that inhibit the enzymatic activity of both COX-1 and COX-2. The nonselective NSAID naproxen at 200 µmol/L did not inhibit the growth of LNCaP cells (Fig. 5*C*). However, it enhanced the growth inhibition seen with 1 and 10 nmol/L calcitriol (~65% inhibition with the combination versus ~48% inhibition with 10 nmol/L calcitriol alone). Similarly, in PC-3 cells (Fig. 5*D*), the nonselective NSAID ibuprofen at 150 µmol/L enhanced the growth inhibitory effect of calcitriol (~74% inhibition with the combination versus ~40% inhibition with 10 nmol/L calcitriol alone) whereas it did not affect cell growth when used alone at this concentration.

Based on extensive dose-response analysis (not shown), we calculated the interaction index (γ) using an isobolar method (33) for each drug combination. This analysis indicated a synergistic (superadditive) effect. The data suggested that ~2 to 10 times lower concentration of each drug is needed when used in combination to achieve the same degree of growth inhibition as achieved by the individual drugs.

Discussion

Calcitriol acts by multiple pathways to inhibit the proliferation of prostate cancer cells (5–14). Our study shows that the regulation

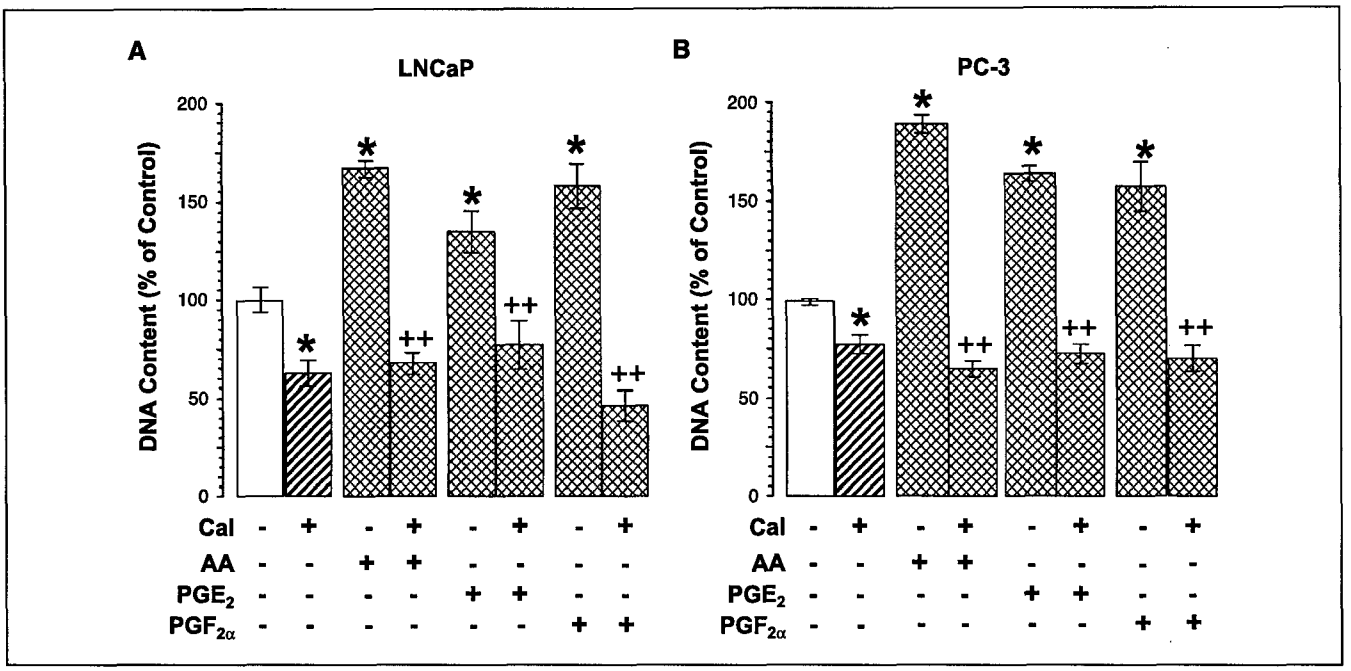

Figure 4. Calcitriol abrogates the growth stimulatory effects of arachidonic acid (*AA*) and exogenous PGs. LNCaP (*A*) and PC-3 (*B*) were treated with arachidonic acid (3 μmol/L), PGE_2 (10 μmol/L), or $PGF_{2\alpha}$ (10 μmol/L) individually or in combination with 10 nmol/L calcitriol (*Cal*) for 6 days. Cell growth was determined by measurement of DNA content as described in Materials and Methods. DNA contents are given as percentage of control value set at 100%, which was equivalent to 12.3 ± 1.2 μg/well for LNCaP cells and 19.3 ± 1.7 μg/well for PC-3 cells. *Columns,* mean from six experiments; *bars,* SE. *, $P < 0.05$, when compared with control; ++, $P < 0.01$, when compared with arachidonic acid, PGE_2, or $PGF_{2\alpha}$ alone.

of PG metabolism is a novel and additional pathway by which calcitriol may exert its antiproliferative actions in prostate cancer cells. We have shown that calcitriol regulates biologically active PG levels and PG actions by three mechanisms: (*a*) the suppression of COX-2 expression, (*b*) the up-regulation of 15-PGDH expression, and (*c*) the reduction of EP2 and FP PG receptor mRNA expression. We propose that these three effects act together to effectively inhibit the stimulation of prostate cancer cell proliferation by endogenously derived PGs as well as PGs added exogenously. Because PGs have been shown to promote prostate cell growth, inhibit apoptosis, and stimulate prostate cancer progression (18–20), we postulate that these effects of calcitriol to reduce PG actions significantly contribute to the anticancer effects of the hormone in prostate cancer.

The transformation of arachidonic acid into PGs and thromboxanes in mammalian cells is catalyzed by the enzyme COX, which has two well-characterized isoforms. COX-1 is constitutively expressed and is involved in housekeeping functions (17, 34). COX-2 is an immediate-early gene that is induced by a variety of growth promoting stimuli such as serum and growth factors, tumor promoters, cytokines, and proinflammatory agents (17, 34), and is regarded as an oncogene (24). COX-2 is overexpressed in various cancers including some, but not all, prostate cancers (18, 21). Inhibitors of COX-2 activity have been shown to suppress prostate cancer cell growth both *in vivo* and *in vitro* (31, 35, 36). Our results show the significant repression of COX-2 mRNA expression by calcitriol in prostate cancer cell lines as well as in primary prostatic epithelial cells and also a reduction in COX-2 protein levels in prostate cancer cell lines, suggesting that COX-2 is a calcitriol target gene.

PGE_2 and $PGF_{2\alpha}$ are rapidly catabolized *in vivo* into their biologically inactive 13,14-dihydro-15-keto metabolites by a two-step process carried out sequentially. The first step is initiated by the reversible oxidation of their 15(*S*)-hydroxyl group by the enzyme 15-PGDH (37). 15-PGDH is widely expressed in many mammalian tissues (38) and has been shown to be modulated by several hormones and factors (37–39), indicating the potential importance of the regulation of this enzyme. In LNCaP cells, 15-PGDH expression is up-regulated by androgens, interleukin-6, and the cyclic AMP inducer forskolin in a protein kinase A–dependent manner (40, 41). We now show that calcitriol is an important regulator of 15-PGDH expression in prostate cancer cells. The partial repression of COX-2 mRNA expression and the increase in 15-PGDH mRNA expression are also seen in primary prostatic epithelial cells derived from normal prostate, suggesting that these calcitriol effects are not restricted to malignant prostate cells. 15-PGDH expression has been shown to be decreased in many cancers (22, 23, 42). Calcitriol has also been shown to increase the expression of 15-PGDH in neonatal monocytes (43), where it exhibits prodifferentiation effects. 15-PGDH, which physiologically antagonizes COX-2, has recently been described as a putative oncogene antagonist that functions as a tumor suppressor in colon cancer by Yan et al. (24) who found that 15-PGDH was universally expressed in normal colon specimens but was routinely absent or severely reduced in cancer specimens. More importantly, stable transfection of a 15-PGDH expression vector into cancer cells greatly reduced the ability of the cells to form tumors and/or slowed tumor growth in nude mice. The authors concluded that 15-PGDH suppressed the effects of the oncogene COX-2 and exhibited an additional effect to inhibit angiogenesis *in vivo* (24). Our present study shows calcitriol-mediated suppression of the oncogene COX-2 and an increase in the expression of the putative tumor suppressor 15-PGDH in prostate cells, suggesting that calcitriol may play an important role in the chemoprevention of prostate cancer.

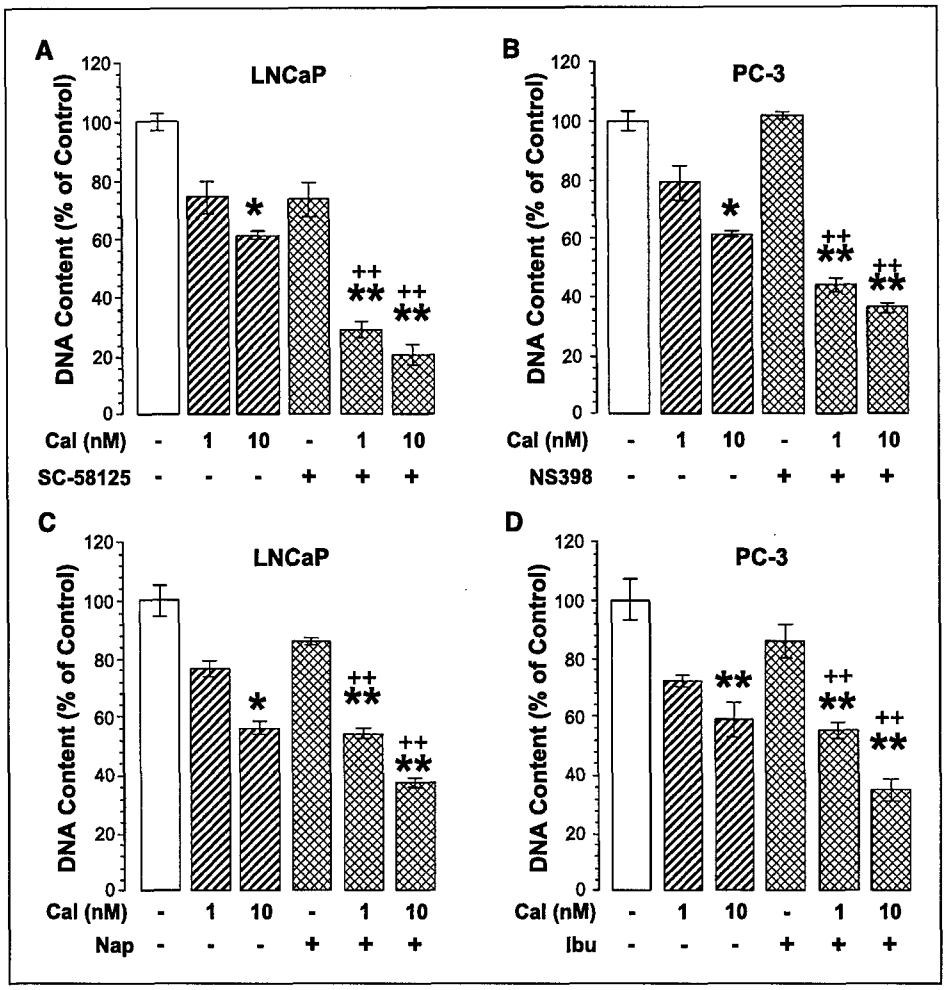

Figure 5. Synergistic inhibition of prostate cancer cell growth by calcitriol and NSAIDs. LNCaP or PC-3 cells were treated with 0.1% ethanol vehicle (*Con*) or 10 nmol/L calcitriol (*Cal*) in the presence and absence of the indicated NSAID. Cell growth was determined by measuring the DNA content as indicated in Materials and Methods. DNA contents are given as percentage of control value set at 100%. *A*, LNCaP cells treated with a combination of calcitriol (*Cal*) and COX-2–specific NSAID SC-58125 (5 μmol/L). 100% DNA content = 10.15 ± 1.22 μg/well. *B*, PC-3 treated with calcitriol (*Cal*) in the presence and absence of the COX-2–selective NSAID NS-398 (7.5 μmol/L). 100% DNA content = 17.42 ± 1.93 μg/well. *C*, LNCaP cells treated with calcitriol (*Cal*) in the presence and absence of the nonselective NSAID naproxen (*Nap*; 200 μmol/L). 100% DNA content = 9.22 ± 0.5 μg/well. *D*, PC-3 cells treated with calcitriol (*Cal*) in the presence and absence of the nonselective NSAID ibuprofen (*Ibu*; 150 μmol/L). 100% DNA content = 21.7 ± 0.9 μg/well. *Columns*, mean from six experiments; *bars*, SE. *, $P < 0.05$; **, $P < 0.01$, when compared with control. ++, $P < 0.01$, when compared with 1 or 10 nmol/L Cal alone.

As a result of its dual action to modulate COX-2 and 15-PGDH expression, we expected calcitriol to reduce the levels of PGs in prostate cancer cells. This indeed was the case as shown by the decrease in PGE_2 levels in the conditioned media from LNCaP cells following calcitriol treatment. Calcitriol regulation of PGE_2 synthesis and secretion has been also reported in growth plate chondrocytes (44), in monocytes (43, 45), and in interleukin-1β–stimulated rheumatoid synovial fibroblasts (46). The effects of calcitriol on PG synthesis and signaling in these target cells seem to be related to the rapid nongenomic actions of calcitriol (47).

PGs exert their myriad effects through G-protein coupled membrane receptors which activate different signal transduction pathways (48). Prostate cancer cells have been shown to express the PGE receptor subtypes EP2 and EP4 (29). Interestingly, our study shows that calcitriol decreases the mRNA expression of the PGE_2 and $PGF_{2\alpha}$ receptor subtypes EP2 and FP, providing yet another mechanism for the suppression of the biological activity of PGs by calcitriol. In a recent study examining the changes in gene expression profile in the kidney of vitamin D receptor (VDR) knockout mice, Li et al. (49) report increases in the expression of EP3 and FP genes in $VDR^{-/-}$ kidneys, suggesting that calcitriol may also regulate the expression of PG receptors in kidney. Our study indicates that calcitriol not only decreases the concentration of PGs but may also inhibit the biological activity of these reduced PG levels by repressing of EP2 and FP receptor mRNA expression in prostate cancer cells.

Chen and Hughes-Fulford (29) have shown that arachidonic acid increases the expression of the immediate-early gene *c-fos* by undergoing a COX-2–mediated conversion to PGE_2, binding of PGE_2 to EP2/EP4 receptors, and subsequent activation of the protein kinase A pathway, which leads to the expression of growth-related genes. PGE_2 has also been to shown to up-regulate the gene expression of its own synthesizing enzyme COX-2 in prostate cancer cells, thereby completing a positive feedback loop (31, 50). We therefore examined the effect of calcitriol treatment on the induction of *c-fos* and cell growth by arachidonic acid in prostate cancer cells and found that calcitriol abolished *c-fos* induction and growth stimulation by arachidonic acid. Our interpretation of these observations is that they reflect both the effect of calcitriol to decrease endogenous synthesis of PGs due to COX-2 suppression and the ability of calcitriol to attenuate the biological activity of the PGs due to 15-PGDH up-regulation and EP and FP receptor down-regulation. The suppression by calcitriol of the growth stimulation by exogenous PG addition is probably due to its ability to enhance PG catabolism through the up-regulation of 15-PGDH expression as well as PG receptor down-regulation.

NSAIDs are known inhibitors of COX activity and have been shown to exhibit growth-suppressive effects in *in vivo* and *in vitro* models of prostate cancer (19, 35, 36, 50–52). The growth inhibitory and proapoptotic actions of NSAIDs are due to their ability to inhibit cyclooxygenase activity to a large degree, although in recent years mechanisms independent of COX-2 inhibition are also believed to

play a role (52). Our data show that the combination of calcitriol with COX-2–selective, as well as nonselective NSAIDs, acts synergistically to reduce the growth of prostate cancer cells. Our hypothesis is that the action of calcitriol at the genomic level to reduce COX-2 expression decreases the levels of COX-2 protein and allows the use of lower concentrations of NSAIDs to inhibit COX-2 enzyme activity, resulting in the enhanced growth inhibition seen with the combination. The potential use of NSAIDs as chemopreventive or therapeutic agents for a variety of malignancies, including prostate cancer, is being intensely investigated (20, 21, 51, 53). We propose that a combination of calcitriol and NSAID might be a useful therapeutic strategy in prostate cancer. The clinical use of NSAIDs has recently become controversial because of the cardiovascular complications associated with the use of high doses of COX-2–selective NSAIDs for prolonged periods of time (54, 55). In comparison with the COX-2–selective inhibitors, the use of a nonselective NSAID such as naproxen has been shown to be associated with decreased cardiovascular adverse effects (56). As shown by our study, an enhancement of growth inhibition is seen when calcitriol is combined with nonselective NSAIDs such as naproxen and ibuprofen. The clinical utility of the calcitriol combination with a nonselective NSAID is therefore worthy of evaluation, especially because the combination allows the use of lower concentrations of calcitriol and the NSAIDs, thereby improving the safety profile of the NSAIDs.

In conclusion, calcitriol acts by three separate mechanisms: decreasing COX-2 expression, increasing 15-PGDH expression, and reducing PG receptor mRNA levels. We believe that these actions contribute to suppress the proliferative stimulus provided by PGs in prostate cancer cells. The regulation of PG metabolism and biological actions constitutes an additional novel pathway of calcitriol action mediating its antiproliferative effects in prostate cells. We propose that a combination of calcitriol and a nonselective NSAID, such as naproxen, might be a useful therapeutic and/or chemopreventive strategy in prostate cancer, as it would achieve greater efficacy and allow the use of lower concentrations of both drugs, thereby reducing their toxic side effects.

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10th PROUTS NECK MEETING ON PROSTATE CANCER

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PATHWAYS MEDIATING THE GROWTH INHIBITORY ACTIONS OF VITAMIN D IN PROSTATE CANCER

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Vitamin D is emerging as an important hormone that affects the development and progression of many malignancies including prostate cancer (PCa). 1,25-dihydroxyvitamin D3 (calcitriol), the active form of vitamin D, inhibits the growth and stimulates the differentiation of PCa cells. We have studied established human PCa cell lines as well as primary cultures of normal or cancer-derived prostatic epithelial cells to elucidate the molecular pathways of calcitriol actions. These pathways are varied and appear to be cell-specific. We have used cDNA microarray analysis to elucidate additional genes regulated by calcitriol in order to identify novel therapeutic targets for the treatment of PCa. Several potentially useful target genes have emerged from these studies. In this talk I will highlight two new target genes, both involved in prostaglandin (PG) metabolism. Accumulating evidence has implicated PGs in stimulating the development of many types of cancer including PCa. PGs have been associated with the progression of PCa, tumor invasiveness and tumor grade. Prostatic PGs are formed by the action of the cyclooxygenase enzyme COX-2. The first step in PG inactivation is mediated by 15-hydroxyprostaglandin dehydrogenase (PGDH). We found that calcitriol down-regulates the expression of COX-2 and up-regulates PGDH. Currently there is much interest in the use of COX-2 inhibitors to prevent and/or treat PCa, due to their ability to inhibit growth and induce apoptosis. Moreover, PGDH has recently been proposed as a tumor suppressor. The actions of calcitriol to induce PGDH and inhibit COX-2, constitute a pathway to reduce and/or remove bioactive PGs thereby diminishing PCa proliferation. Treatment of LNCaP cells with a combination of calcitriol and COX-2 inhibitors resulted in synergistic growth inhibition. In combination, calcitriol and COX-2 inhibitors allowed the use of reduced doses of both drugs that still resulted in enhanced antiproliferative activity. These findings suggest that therapy combining calcitriol and COX-2 inhibitors will increase the efficacy of both drugs while decreasing their side-effects. We propose that this combination of already approved drugs can be brought to clinical trial swiftly, particularly in patients with early recurrent PCa that demonstrate rising PSA after primary therapy. In conclusion, our research is directed at understanding the mechanisms of vitamin D action in prostate cells with the goal of developing treatment strategies to improve PCa therapy. The ability of calcitriol to inhibit PG synthesis and stimulate PG inactivation appears to be an additional pathway by which calcitriol can enhance PCa therapy.

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ABSTRACTS OF THE SECOND INTERNATIONAL SYMPOSIUM ON VITAMIN D ANALOGS IN CANCER PREVENTION AND THERAPY

7-8 May 2005, Lübeck, Germany

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amount of UV radiation from natural sunlight and the exposure of only small parts of the body, *e.g.* hands, forearms and/or face, are necessary. The aim of this study was to evaluate the correlation between Vitamin D-weighted UV dosage (H_{vd}) *versus* the increase of circulating $25(OH)D_3$ and $1,25(OH)_2D_3$. *Patients and Methods*: Twenty-two dialysis patients were partial body (frontal part of the legs, approx. 15% of body surface) irradiated over a period of 14 weeks using an artificial UV-source (UVB 3.5%); blood samples were taken every two weeks. *Results*: The peak value of $25(OH)D_3$ was found after 8 weeks (increase Δ + 13µg/l = +33%, median) and the peak of $1,25(OH)_2D_3$ followed 6 weeks later (increase Δ + 9ng/l = +90%) Therefore, the following algorithm can be calculated: $25(OH)_2D_3 = (H_{vd}^2 \times 10^5)+25$, as a nonlinear correlation (r^2=0.992); following a linear correlation (r=0.32) between $25(OH)D_3$ and $1,25\ (OH)_2D_3$. *Conclusion*: A sufficient pool of circulating $25(OH)D_3$ is necessary for conversion to $1,25(OH)_2D_3$. In renal patients, the threshold level of $25(OH)D_3$ seems to be ≥35 µg/l.

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EFFECT OF UVB RADIATION EMITTED FROM THE NARROWBAND TL-01 LAMP (311 NM) ON CALCITRIOL SYNTHESIS IN ORGANOTYPIC CULTURES OF KERATINOCYTES

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The skin is the only tissue known in which the complete UVB-induced pathway from 7-dehydrocholesterol (7-DHC) to hormonally-active calcitriol (1α,25 dihydroxyvitamin D_3) occurs under physiological conditions. It is well known that both calcitriol and UVB radiation exert potent antipsoriatic effects. We speculate that the therapeutic effect of UVB radiation can be attributed to UVB-triggered cutaneous synthesis of calcitriol, for which the optimum wavelength was 300 ± 3 nm *in vitro* and *in vivo*. On the other hand, the narrowband Philips TL-01 lamp, which is commonly used as a UVB source for the treatment of psoriasis, has a maximum spectral irradiance at around 311 nm. The aim of this study was to investigate the calcitriol-inducing potential of the TL-01 lamp in organotypic cultures of keratinocytes supplemented with 25 µM 7-DHC at different radiant exposures (125-1000 mJ/cm^2). We found that the maximum calcitriol-generating capacity of the TL-01 lamp at 500 mJ/cm^2 (corresponding to 2.1 SED [Standard Erythema Dose]) and 16 hours after irradiation still amounted to approximately 45% of that of monochromatic radiation at 300 nm and 30 mJ/cm^2. We conclude that irradiation with the narrowband TL-01 lamp in a therapeutic dose range can affect calcitriol synthesis in epidermal keratinocytes. Thus, the antipsoriatic effect observed after TL-01 lamp exposures may be, at least partially, explained by the known action of newly-synthesized calcitriol on epidermal cell proliferation and differentiation.

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PATHWAYS MEDIATING THE GROWTH INHIBITORY ACTIONS OF VITAMIN D IN PROSTATE CANCER

Jacqueline Moreno, Aruna Krishnan and David Feldman

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Vitamin D is an important hormone that affects the incidence and progression of many malignancies including prostate cancer (PCa). 1,25-dihydroxyvitamin D (calcitriol), the active form of vitamin D, inhibits the growth and stimulates the differentiation of PCa cells. We studied established human PCa cell lines as well as primary cultures of normal or cancer-derived prostatic epithelial cells to elucidate the molecular pathways of the action of calcitriol. These pathways are varied and some appear to be cell-specific. We used cDNA microarray analysis to ascertain additional genes regulated by calcitriol, in order to identify novel therapeutic targets for the treatment of PCa. Several potentially useful target genes have emerged from these studies including two new target genes, both involved in prostaglandin (PG) metabolism.

Accumulating evidence has implicated PGs in stimulating the development of many types of cancer including PCa. PGs have been associated with the progression of PCa, tumor invasiveness and tumor grade. Prostatic PGs are formed by the action of the cyclooxygenase enzyme COX-2. The first step in PG inactivation is mediated by 15-hydroxyprostaglandin dehydrogenase (PGDH). We found that calcitriol down-regulates the expression of COX-2 and up-regulates PGDH. There is much current interest in the use of second-generation COX-2 inhibitors or non-selective nonsteroidal anti-inflammatory drugs (NSAIDs), to prevent and/or treat PCa, due to their ability to inhibit growth and induce apoptosis. Moreover, PGDH has recently been proposed as a tumor suppressor. The actions of calcitriol to induce PGDH and inhibit COX-2 constitute a pathway to reduce and/or remove active PGs, thereby diminishing PCa proliferation. Combination therapy of LNCaP cells with calcitriol and NSAIDs revealed synergistic growth inhibition. In combination, calcitriol and NSAIDs allowed the use of

reduced doses of both drugs that still resulted in enhanced antiproliferative activity. These findings suggest that therapy combining calcitriol and NSAIDs will increase efficacy while decreasing side-effects. We propose that this combination of already approved drugs can be brought to clinical trial swiftly, particularly in patients with early recurrent PCa that demonstrate rising PSA after primary therapy. In conclusion, our research is directed at understanding the mechanisms of vitamin D action in prostate cells with the goal of developing treatment strategies to improve PCa therapy. The ability of calcitriol to inhibit PG synthesis and stimulate PG destruction appears to be an additional pathway by which calcitriol can enhance PCa therapy.

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BIOLOGICAL EFFECTS OF 1α,25-DIHYDROXYVITAMIN D_3 ON HUMAN KERATINOCYTES AFTER IONIZING RADIATION

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Exposure of human skin to ionizing radiation results in various early and late effects such as an inflammatory reaction, keratosis, fibrosis, radiation vasculitis and cancer. 1α,25-Dihydroxyvitamin D_3, the biologically active metabolite of vitamin D, has been shown to exert pleiotropic effects in the skin. We evaluated whether the radiation reaction of human keratinocytes (HaCaT cells) can be modulated by 1α,25-dihydroxyvitamin D_3. The cell growth of keratinocytes after ionizing radiation was significantly increased in the presence of 1α,25-dihydroxyvitamin D_3 as compared to the untreated control. Moreover, 1α,25-dihydroxyvitamin D_3 also exerted a positive influence on the cell survival of irradiated keratinocytes, as shown by clonogenic assay. As the cutaneous radiation reaction is determined by various inflammatory parameters, including adhesive interactions mediated by cellular adhesion molecules, we analyzed the cell surface expression of intercellular adhesion molecule-1 (ICAM-1) and β1-integrin in keratinocytes and the effect of 1α,25-dihydroxyvitamin D_3 using flow cytometry and immuno- histochemistry. The results revealed that ionizing radiation causes an up-regulation of both ICAM-1 and β1-integrin in keratinocytes, which was inhibited by pretreatment of the cells with 1α,25-dihydroxyvitamin D_3. Taken together, our data suggest that 1α,25-dihydroxyvitamin D_3 might be a promising agent to modify the radiation reaction, offering new options in radiotherapy and oncology.

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SNAIL REPRESSES VITAMIN D RECEPTOR EXPRESSION AND BLOCKS THE EFFECTS OF 1α,25-DIHYDROXYVITAMIN D_3 ON HUMAN COLON CANCER CELLS *IN VITRO* AND *IN VIVO*

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We have previously reported that 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) and several non-hypercalcemic analogs (EB1089, MC903 and KH1060) inhibit proliferation and promote differentiation of human SW480-ADH colon cancer cells. They induce the expression of E-cadherin and the translocation of β-catenin from the nucleus to the plasma membrane. The Wnt/β-catenin signaling pathway is deregulated in most colon cancers as a result of mutation of APC or β-catenin (*CTNNB1*) genes. In several human colon cancer cell lines analyzed, 1α,25$(OH)_2D_3$ repressed β-catenin/TCF-4 transcriptional activity and thus inhibited the expression of β-catenin/TCF-4-responsive genes. Using oligonucleotide microarrays, the genetic profile induced by 1α,25$(OH)_2D_3$ in human colon cancer cells was identified. 1α,25$(OH)_2D_3$ changed the expression levels of numerous previously unreported genes, including many involved in transcription, cell adhesion, DNA synthesis, apoptosis and intracellular signaling. Vitamin D receptor (VDR) is expressed in normal colon epithelium and during the early stages of colon cancer, but is lost at later stages of tumor progression. High VDR expression has been associated with good prognosis. We found that the SNAIL transcription factor represses human *VDR* gene expression in colon cancer cells and blocks the antitumor action of EB1089 in xenografted mice. In human colon cancer, elevated SNAIL expression correlates with the down-regulation of *VDR* and E-cadherin. Our data predict that colon cancer patients with high levels of SNAIL are likely to be poor responders to therapy with 1α,25$(OH)_2D_3$ analogs.

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HIGH-DOSE PULSE CALCITRIOL IN PROSTATE CANCER

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In pre-clinical models of prostate cancer, calcitriol, the principal active metabolite of vitamin D, displayed significant antineoplastic activity alone and in combination with cytotoxic drugs, but only at substantially supraphysiologic concentrations. The reported mechanisms of activity include inhibition of proliferation and cell cycle

PROGRAM & ABSTRACTS

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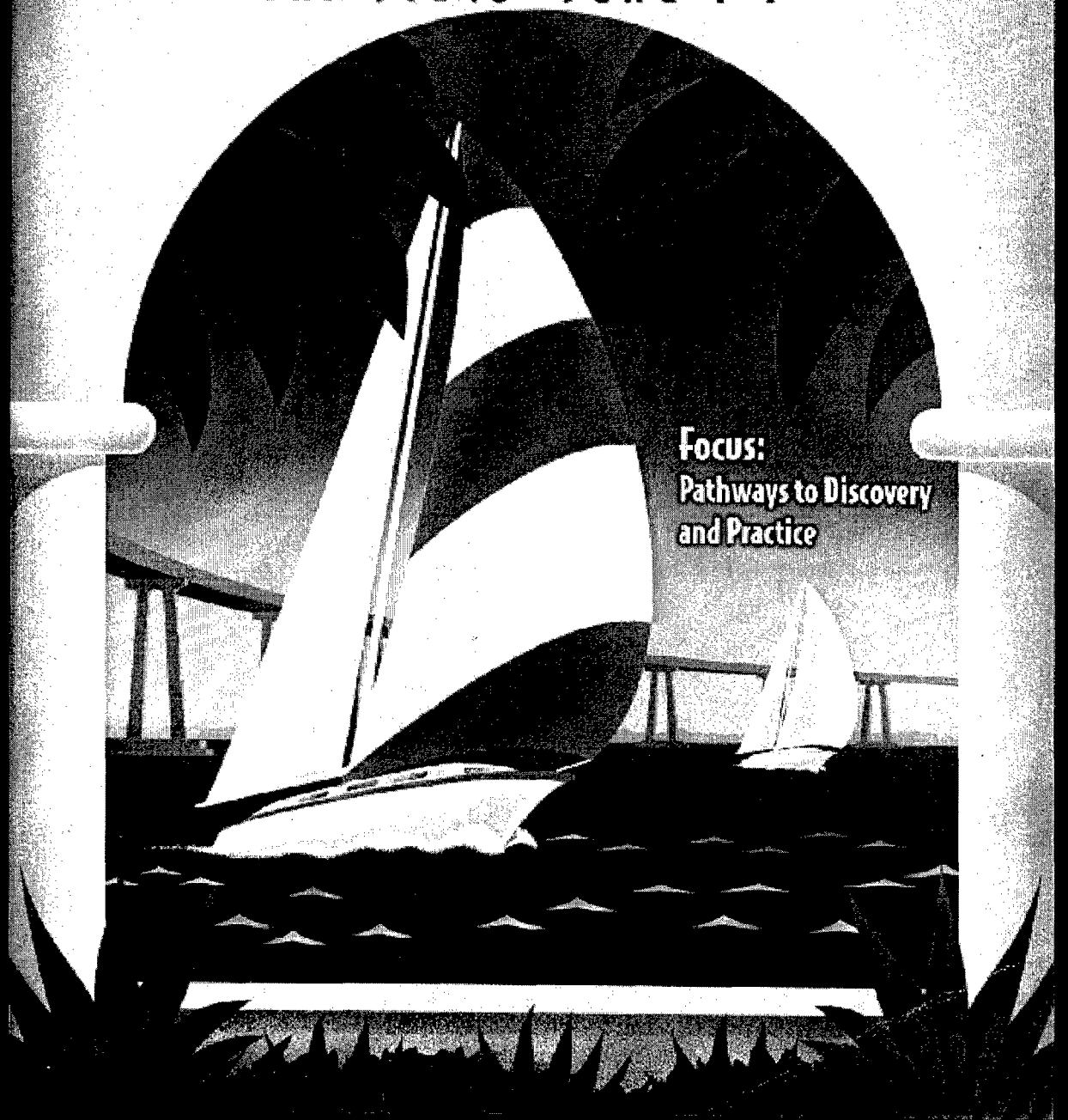

THE ENDOCRINE SOCIETY'S 87TH ANNUAL MEETING
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triggered meiosis? To address these issues, we have further characterized the steroid binding and signaling properties of the classical Xenopus progesterone and androgen receptors. We demonstrate that the Xenopus progesterone receptor (XPR-1) binds to and is transcriptionally activated by many androgens, including androstenedione, testosterone, and dihydrotestosterone, at 100 - 200 nM concentrations. Furthermore, the Xenopus androgen receptor (XeAR) binds to and is activated by progesterone at even lower concentrations (1 - 20 nM). Interestingly, the traditional mammalian PR antagonist RU486 is a poor inhibitor of progesterone binding to XPR-1 (IC50=1 nM), but a potent inhibitor of androgen binding to XeAR (IC50=5 nM). The inability of RU486 to significantly block progesterone interactions with XPR-1 is likely due to a cysteine at position 376 in the ligand binding domain, as most RU486-sensitive PRs have a glycine residue at this position. Accordingly, XeAR contains a glycine residue in the corresponding position, which might explain its sensitivity to RU486. Together, these results reconcile the discrepancies regarding which classical receptors are regulating progesterone and androgen-triggered *Xenopus laevis* oocyte maturation, as both receptors are likely activated under either condition, but RU486 and androgen receptor antagonists will only block XeAR-mediated effects.

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P2-657

Androgen Receptor Down-Regulation by the Antiestrogen ICI 182,780 (Faslodex) in LNCaP Prostate Cancer Cells.

Rumi S Bhattacharyya*[1], Aruna V Krishnan[1], Srilatha Swami[1], David Feldman[1], *[1]Dept of Med, Endocrinol Div, Stanford Univ Sch of Med, Stanford, CA.*

For prostate cancer (PCa) patients not cured by primary therapy, androgen deprivation therapy (ADT) is often successful in causing PCa regression since these cancers are generally androgen-dependent. Unfortunately, most men eventually fail ADT and their disease transforms to an androgen-independent PCa (AIPC), for which there is no effective treatment. In AIPC, most PCa cells still retain expression of the androgen receptor (AR) which plays a role in the continued growth of the cancer. We hypothesize that drugs that are Selective AR Down-Regulators (SARDs) present a unique approach for the treatment of AR-dependent AIPC. Decreasing the AR concentration will significantly reduce PCa growth stimulation. Our studies have demonstrated that the antiestrogen, ICI 182,780 (ICI) (Faslodex) has SARD activity in LNCaP PCa cells. Treatment of LNCaP cells with ICI (10 nM) resulted in a 50% decrease in AR protein expression after 48 hrs as measured by [^{3}H]-DHT binding and Western blot analysis. ICI also decreased AR mRNA levels. Decreased AR mRNA expression was observed as early as 6 hrs after ICI treatment, the maximal decrease (60% of control) being seen at 24 hrs and by 48 hrs the inhibitory effect of ICI was diminished. Preliminary experiments aimed at understanding the mechanism of AR down-regulation by ICI suggest that it is occurring at the transcriptional level. The AR present in LNCaP cells contains a point mutation that renders it "promiscuous", allowing non-androgen ligands to bind to the receptor. In competition binding assays, increasing concentrations of ICI did not displace [^{3}H]-DHT binding to the AR, demonstrating that ICI did not bind to the mutant AR at the DHT binding site. AR down-regulation by ICI resulted in decreased AR-mediated functional responses as measured by PSA secretion and PSA mRNA expression. ICI inhibited R1881 stimulated PSA secretion by 60-70% after a 6 day treatment. PSA mRNA expression was decreased by 30-40% after 24 hrs of ICI and R1881 co-treatment when compared to R1881 alone. Importantly, ICI caused significant inhibition of LNCaP cell growth in a time and dose-dependent manner. At the end of 6 days of treatment a 60% growth inhibition was seen in ICI-treated cells compared to control. These data demonstrate that the anti-estrogen ICI is a potent AR down-regulator which causes significant inhibition of PCa cell growth. SARDs, such as ICI, present viable new options for treating AR-dependent advanced PCa.

P2-658 ⊗

Regulation of Prostaglandin Metabolism by Calcitriol: Potential Role in the Treatment of Prostate Cancer.

Jacqueline Moreno*[1], Aruna V Krishnan[1], David Feldman[1], *[1]Dept of Med, Stanford Univ Sch of Med, Stanford, CA.*

Calcitriol exhibits growth inhibitory and pro-differentiation effects in *in vitro* and *in vivo* models of prostate cancer (PCa). Our goal is to define the mechanisms underlying the antiproliferative effects of calcitriol in PCa. cDNA microarray analysis of LNCaP human PCa cells showed the regulation of the expression of 28 genes by calcitriol. Interestingly, two of these genes are involved in the metabolism of prostaglandins (PGs), known stimulators of PCa cell growth. The expression of PG synthesizing cyclooxygenase-2 (COX-2) gene was significantly decreased by calcitriol, and that of PG inactivating 15 prostaglandin dehydrogenase (15-PGDH) was increased by calcitriol. This dual action of calcitriol would reduce the levels of biologically active PGs in PCa cells, thereby decreasing their proliferative stimulus. In the current study we showed that calcitriol increased 15 PGDH mRNA and protein levels in LNCaP cells in a time and dose-dependent manner. The increase in 15-PGDH mRNA expression reached a peak after 6 h of calcitriol treatment that was maintained over 36 h. Calcitriol reduced COX-2 mRNA to approximately 50 % of control in both LNCaP and PC-3 cells and decreased the concentration of COX-2 protein in PC-3 cells. We observed a 60 % decrease in levels of PGs in the conditioned media of LNCaP cells with calcitriol. We believe this decline is the result of the dual effect of calcitriol on the expression of PG metabolic enzymes. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX-2 and exhibit antitumor effects in both *in vitro* and *in vivo* PCa models. As a down-regulator of COX-2 expression, calcitriol would enhance COX-2 inhibition when combined with an NSAID. We found that the combination of calcitriol with NSAIDs acted synergistically to reduce the growth of LNCaP and PC-3 cells, exhibiting up to 50% more growth inhibition with the combination than induced by NSAIDs or calcitriol alone. The combination was also more effective than individual drugs in reducing PG secretion by LNCaP cells. The ability of calcitriol to inhibit PG synthesis and stimulate PG catabolism is an additional pathway by which calcitriol exerts its antiproliferative actions. The therapeutic combination of calcitriol and NSAIDs would allow the use of lower concentrations of either drug, reducing their toxic side-effects. We propose that calcitriol and NSAID combination might be a useful therapeutic strategy in men with early recurrent PCa.

P2-659

Direct Regulation of Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3) by Androgens and Calcitriol in LNCaP Human Prostate Cancer Cells.

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Calcitriol exhibits growth inhibition and differentiation in a variety of cell types including prostate cancer cells (PCa). In the androgen dependent LNCaP human PCa cell line calcitriol exerts its antiproliferative action predominantly by inducing cell cycle arrest. Previously we have shown that growth arrest is mediated primarily by induction of IGFBP-3, which subsequently increases the expression of the cell cycle inhibitor p21. We have identified a functional vitamin D response element (VDRE) in the IGFBP-3 promoter directly mediating the induction by calcitriol. In this report we show that androgens increase expression of IGFBP-3 at both mRNA and protein levels in LNCaP cells. Furthermore, the combination of calcitriol and androgens results in a substantial increase in IGFBP-3 indicating a strong synergistic effect of calcitriol and androgens on IGFBP-3 expression. To understand the molecular mechanism involved, we examined the IGFBP-3 promoter for interactions between calcitriol and androgens. Transactivation assays show that the 6 kb IGFBP-3 promoter sequence responds to androgen treatment. A time course of IGFBP-3 mRNA expression in LNCaP cells treated with various concentrations of the synthetic androgen R1881 also suggests that androgen directly regulate the transcription of IGFBP-3 in a dose-dependent manner. A series of deletions generated within the 6 kb promoter demonstrated that the ARE is present in the DNA fragment between -3753 and -3500. Point mutations in the potential ARE resulted in a loss of androgen induction confirming the critical response element sequences. Furthermore, chromatin immunoprecipitation assays showed that R1881 treatment recruited the androgen receptor to the ARE site in the IGFBP-3 promoter in intact cells. In addition, the combination treatment of androgens and calcitriol doubles the effect of either calcitriol or androgens alone on the IGFBP-3 promoter constructs. In conclusion, the functional VDRE and ARE in the IGFBP-3 promoter directly mediate the interaction of calcitriol and androgens on IGFBP-3 expression. It may be counter-intuitive that androgens stimulate a factor mediating antiproliferative and proapoptotic actions on PCa cells. However, a number of studies show that androgens mediate an antiproliferative and prodifferentiative action on PCa cells. The clinical significance of these findings will require further study.

P2-660 ♦

Co-Treatment of Human Prostate Cancer Cells with S179D Prolactin and 1,25 dihydroxy Vitamin D3 (VD) Produces Synergy in the Promotion of Apoptosis and Brings the Dose of VD Required into the Non-Toxic, Physiological Range.

Wei Wu*, Aimee M Walker*, *[1]Biomed Sci, Univ of California, Riverside, CA.*

S179D prolactin (PRL) is a molecular mimic of phosphorylated human PRL that inhibits the growth of human prostate cancer cells when these are grown in vitro or as tumors in nude mice (1). In this study, we have investigated the interplay between S179D PRL and another inhibitor of prostate cancer growth, 1, 25 dihydroxy vitamin D3 (VD). When prostate cancer cells were incubated in S179D PRL for 3 days, doses up to 40 nM had no effect on cell number, although longer incubations were very effective at 10 nM. In the 3-day time frame, incubations up to 160 pM VD were also ineffective at reducing cell number, whereas those between 30 and 120 nM were effective. Incubation in S179D PRL at 20 nM (DU145 cells) or 10 nM (PC3 cells) sensitized the cells to VD such that a 50% reduction in cell number occurred at 60 pM and 100 pM VD in DU145 and PC3 cells, respectively. When DNA degradation was assessed, S179D PRL alone (same concentrations) and VD alone (300 pM) had no effect, but together there was marked DNA degradation. The 3-day incubation in either S179D PRL or VD produced no significant increase in the amount of the VD receptor (VDR), whereas co-incubation in S179D PRL and VD doubled the amount of the VDR, as assessed by Western blot. The same incubation conditions resulted in a doubling of p21 protein in response to S179D